32
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CONTENTS

Studies on Johnin—Howard W. Johnson and Benjamin F. Cox..... 131

Some Observations on the Pathology of Pneumonia in the Food-Producing Animals—Robert F. Langham, Frank Thorp, Jr., Ronald T. Ingle and L. B. Sholl..... 139

Studies on the Allergic and Antigenic Activity of Sonic Filtrate of Brucella Abortus—E. L. Stubbs and I. Live..... 146

Feeding as a Contributory Factor in the Development of Chronic Mastitis—Earl N. Moore, H. O. Henderson, A. H. Van Landingham and Chas. E. Weakley, Jr. 154

The Relation of Leucemia and Bovine Lymphocytoma—W. W. Thompson and Lee M. Roderick..... 159

Listerella Infection in Cattle—L. H. Schwarte and H. E. Biester..... 165

Epithelial Tumors of Horses—Russell A. Runnells and E. A. Benbrook.... 176

Neutralization Studies of the Viruses of Influenza A, Influenza B and Swine Influenza with Equine Influenza Convalescent Serums—T. C. Jones and Fred D. Maurer..... 179

Transmission of Equine Infectious Anemia by the Stablefly, Stomoxys Calcitrans, the Horsefly, Tabanus Sulcifrons (Macquart), and by Injection of Minute Amounts of Virus—C. D. Stein, J. C. Lotze and L. O. Mott. 184

Notes on Susceptibility of the Golden Hamster (Cricetus Auratus) to Equine Encephalomyelitis Virus—M. S. Shahan and G. T. Creech... 193

Observations on a Fungus Skin Disease of Iowa Muskrats—Paul L. Errington 195

The Effect of Artificial Cryptorchidism on the Prostate Gland of Dogs—Carl F. Schlotthauer and Jesse L. Bollman..... 202

A Filtrable Virus, the Cause of a Respiratory-Nervous Disorder of Chickens—Donald E. Stover..... 207

Further Observations on a Blood Protozoan of Turkeys Transmitted by Simulium Nigroparvum (Twinn)—E. P. Johnson..... 214

An Electrophoretic Study of Pullorum-Agglutinating Chicken Serums—Charles L. San Clemente..... 219

Ineffectiveness of Cow Urine in the Control of Eimeria Tenella Infection in Chickens—Herbert Fisher, Cameron Inkster and Jacob Biely..... 221

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VOL. III APRIL 1942 NO. 7

Studies on Johnin*

HOWARD W. JOHNSON, B.S., M.S., D.V.M., and BENJAMIN F. COX, B.S.
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I. The Comparative Sensitivity of Rabbits, Guinea Pigs and Chickens Inoculated with Mycobacterium Paratuberculosis

AS A RESULT of studies made by injecting rabbits, guinea pigs and chickens with saline suspensions of *Mycobacterium paratuberculosis*, Twort[1] and others concluded that those animals were highly resistant to infection with this organism and, therefore, unsatisfactory for the study of Johne's disease. The results obtained by the authors confirmed these findings. Rabinowitch,[2] Grassberger[3] and Hagan and Levine[4] showed that saprophytic acid-fast bacilli injected intraperitoneally in fairly large doses, with butterfat, lanoline or mineral oil as a suspending medium, may give rise to extensive lesions in guinea pigs, rabbits and mice. Mohler,[5] using large injections of mineral oil suspensions of *M. paratuberculosis*, demonstrated similar lesions in guinea pigs, rabbits, rats, mice, pigeons and chickens.

As far as we are aware, no attempt has been made to study and compare the sensitivity produced by injecting rabbits, guinea pigs and chickens with an oil suspension of *M. paratuberculosis*. In this connection, studies were made to answer the following questions: Are the animals sensitized by this inoculum? In a group of the same species, how uniform is the sensitivity? Is the duration of sensitivity the same in the different species? Which species will be the most satisfactory test animals?

It is the object of this paper to present some detailed studies on the use of mineral oil suspensions of *M. paratuberculosis* for sensitizing rabbits, guinea pigs and chickens.

MATERIALS AND METHODS

The johnins used in these experiments were made from synthetic culture mediums on which *M. paratuberculosis* had grown satisfactorily. The method employed to prepare johnin from mature cultures of this organism was the same as that used by Dorset and Henley[6] to prepare tuberculin from cultures of *M. tuberculosis*.

The materials for sensitizing were prepared as follows: Cultures of *M. paratuberculosis* were planted on the surface of synthetic liquid culture mediums and allowed to grow for at least one month. The resulting organisms were separated from the mediums by filtration through sterile Seitz filters. After all of the liquid had been drawn through the filter, a slight vacuum was maintained for about ten minutes to partially dry the organisms. This facilitated their suspension in oil later. The partially dried mass was weighed and placed in a sterile, covered mortar. For each gram of organisms 10 cc. of mineral oil was added slowly with constant stirring. This suspension was poured into vials and shaken in a shaking machine for one hour.

White guinea pigs and rabbits were chosen for these experiments because the reactions to the intradermic test are much more readily interpreted in light-colored skin. White Leghorns

*From the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, U. S. Department of Agriculture.

[1]Twort, F. W., and Ingram, F. L. Y.: A monograph on Johne's disease (Baillière, Tindall and Cox, London, 1913).

[2]Rabinowitch, L.: Zeit. f. Hyg. u. Infekt., xxvi (1897), p. 90.

[3]Grassberger, R.: Münch. Med. Wochnschr., xlvi (1899), pp. 341, 382.

[4]Hagan, W. A., and Levine, P.: J.A.V.M.A., lxxxi (1932), p. 723.

[5]Mohler, W. M.: J.A.V.M.A., xciv (June 1939), p. 590.

[6]Dorset, M., and Henley, R. R.: A synthetic medium for *B. tuberculosis* with a description of the method of producing tuberculin. Biochemic Division, U. S. Bureau of Animal Industry (Nov. 7, 1934).

TABLE I—Results of Repeated Tests on Sensitized Guinea Pigs (Intraperitoneal Inoculation)

Weeks After Inoculation	No. Tests	Typical Reactions	% Typical Reactions	Death Shock	% Death Shock	Negative Reaction	% Negative Reaction
4	123*	76	61.7	11	8.9	36	29.2
6	69	38	55.0	6	9.3	25	36.2
8	63	21	33.3	16	26.6	26	41.2
12	47	14	29.7	7	13.4	26	55.3
20	25	8	32.0	2	25.0	15	60.0
Total	327	157	47.7	42	12.8	128	39.1

*Two guinea pigs died before the initial test.

were chosen because the large and early maturing wattles, which are characteristic of this breed, are convenient for the intradermic injection of johnin and for the reading of reactions.

A number of rabbits and guinea pigs were inoculated intraperitoneally and other groups of the same species were inoculated subcutaneously. Chickens were inoculated either as embryos or as young chicks.

Experimental Data

Rabbits.—Twenty-two rabbits, averaging slightly less than 1 kg., were inoculated each with 0.2 Gm., moist weight, of *M. paratuberculosis* culture suspended in 2 cc. of mineral oil. Sixteen rabbits were inoculated intraperitoneally and six subcutaneously. Each of the 22 rabbits was tested intradermically with 0.1 cc. of johnin, four to twelve weeks after the sensitizing inoculation.

Four weeks following the sensitizing inoculation, the johnin test on the 16 intraperitoneally injected rabbits elicited five typical reactions and failed to produce reactions in eleven rabbits. A retest on the same animals with the same test material, twelve weeks after the sensitizing inoculation, caused very slight response. The reaction at this time was characterized by a slight thickening of the skin at the point of injection.

The six subcutaneously inoculated rabbits were tested intradermically with johnin four weeks following the sensitizing inoculation; three showed typical reactions and three failed to react. A retest twelve weeks following the sensitizing inoculation failed to elicit a response in a single rabbit.

It appears therefore, that the sensitization in rabbits inoculated either subcutaneously or intraperitoneally with an oil suspension of *M. paratuberculosis* was of short duration and not uniform as shown by the intradermic johnin test. The absence of gross lesions and the almost complete lack of sensitization would seem to indicate that

TABLE II—Results of Johnin Tests and Postmortem Examinations on Chickens Inoculated as 5-Day Embryos* with 0.2 cc. of Oil Suspension of M. Paratuberculosis.

Test Materials	Date	Bird Number																						
		53	54	52	50	44	51	45	30	57	55	56	33	49	47	46	42	43	38	39	31	32	34	35	36
Johnin 110	8- 8-39	+	+	+	+	+	—	S	S	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Johnin 110	8-22-39	+																							
Johnin 110	9- 5-39	+	+																						
Johnin W	9-19-39	+	+	—																					
Johnin W	10- 3-39	+	+	—	+																				
Johnin W	10-17-39	+	+	—	—	+	S	—	—	—	S														
Johnin 3A-1	10-31-39	S	+	S	S	+	S																		
Johnin 1-A	11-14-39	+	+	—	S	+	—																		
Johnin 9A	1- 3-40	S	—	—	S	+	+																		
Destroyed	1-20-40																								
P. M. lesions		—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Acid-fast smears		—	—	—	—	+	—	+	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—	—

*Hatched 5-3-39.
+ = Positive.
S = Slight.
— = Negative.
P.M. = Postmortem.

the rabbits used by this laboratory were extremely resistant to *M. paratuberculosis* and were, therefore, unsatisfactory laboratory animals for the study of this organism.

Guinea Pigs.—Four hundred and twelve guinea pigs, weighing from 250 to 350 Gm., were inoculated intraperitoneally each with 0.05 Gm. of *M. paratuberculosis* culture, suspended in 0.5 cc. of mineral oil. Twenty-eight additional guinea pigs were inoculated subcutaneously with a similar inoculum. All intradermic tests were made by injecting 0.1 cc. of johnin in the shaved skin over the abdomen.

Two hundred and eighty-seven of the 412 intraperitoneally inoculated guinea pigs were tested four weeks after the sensitizing inoculation. Typical reactions, ranging from 1 to 3 cm. in diameter, with marked edema, erythema, and with 0.5 to 1 cm. of central necrosis, were obtained in 157 (54.7%) of the animals; 31 (10.8%) died as the result of shock; no reactions were elicited in 99 (34.4%).

To study the effect of repeated intradermic tests on intraperitoneally inoculated guinea pigs, 5 groups of 25 animals each were inoculated intraperitoneally, each group with a different strain of *M. paratuberculosis*. The results of this test are summarized in table I. The increase in the percentage of non-reactors and the decrease in the percentage which showed typical reactions were due largely to the high mortality in the reactor group.

All 28 subcutaneously inoculated guinea pigs showed typical reactions to the initial intradermic johnin test four weeks after the sensitizing inoculation. However, a second intradermic johnin test, eight weeks after the sensitizing inoculation, elicited only three (10.7%) typical reactions.

Experimental results indicate that the guinea pig is more satisfactory than the rabbit as a laboratory animal for studying *M. paratuberculosis* as shown by the more extensive lesions, the higher percentage of positive reactions and a greater degree of sensitization. It should be noted, however, that the guinea pigs sensitized by intraperitoneal inoculations are unsatisfactory

TABLE III—Results of Johnin Tests and Postmortem Examinations on Chickens Inoculated as 10-Day Embryos* with 0.2 cc. of Oil Suspension of M. Paratuberculosis

| Test Material | Date | Bird Number | | |

TABLE IV—Results of Johnin Test and Postmortem Examinations on Chickens Inoculated as Day-Old Chicks* with 0.2 cc. of Mineral Oil Suspension of M. Paratuberculosis

Test Materials	Date	Bird Number																			
		138	139	124	125	126	127	128	140	133	141	135	122	121	130	123	137	120	129	132	134	142
Johnin 110	8- 8-39	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Johnin 110	8-22-39	+																				
Johnin 110	9- 5-39	+	+																			
Johnin 110	9-19-39	+	+	—																		
Johnin 110	10- 3-39	S	+	S	+														—			
Johnin 110	10-17-39	—	+	S	+	+																
Johnin 3A-1	10-31-39	S	—	+	+	—	+															
Johnin 1A	11-14-39	—	+	+	+	+	+	+														
Johnin 9A	1- 3-40	+	+		+	S	+	+	+	+	+											
Johnin 4F	2-13-40	—	+		+	S	+	+		+	+	+	+	S	+	+	+					
Destroyed	2-20-40																					
P. M. lesions		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+	—
Acid-fast smears		+	+	—	—	+	—	+	—	+	+	+	+	—	+	+	+	—	+	—	+	—

*Hatched 5-3-39. — = Negative.
+ = Positive. P.M. = Postmortem.
S = Slight.

for comparative studies of johnins because of the variation in the size of the reactions, deaths from shock and the relatively high percentage of non-reactors. The uniformity in positive reactions brought about by the use of subcutaneous injections for sensitizing guinea pigs seems to afford a more satisfactory method for a comparative study of the relative sensitivity to johnins, but the sensitivity is only transitory.

Chickens.—Two methods were used in the search for a satisfactory procedure for sensitizing chickens: (1) the injection of a mineral oil suspension of *M. paratuberculosis* into 5- and 10-day-old developing chick embryos and (2) the injection of similar suspensions intraperitoneally into day-old and month-old chickens.

The eggs were incubated in an electric chick incubator and candled on the fifth and tenth days of incubation. The living embryos were inoculated through an opening drilled into the shell, large enough to admit a 20-gauge needle. The oil suspension of *M. paratuberculosis* was inoculated (0.1- and 0.2-cc. quantities) and the opening sealed with "parafilm." Incubation was resumed until hatching occurred.

All chickens used in these experiments were tested one or more times with 0.1 cc.

TABLE V—Results of Johnin Tests and Postmortem Examinations on Chickens Inoculated as One-Month-Old Chickens* with 0.2 cc. of Mineral Oil Suspension of M. Paratuberculosis

Test Materials	Date	Bird Number											
		101	102	104	98	111	95	96	100	118	114	117	105†
Johnin 110	8- 8-39	+	+	+	+	+	—	—	S	—	+	+	—
Johnin 110	8-22-39	+											
Johnin 110	9- 5-39	+	+										
Johnin 110	9-19-39	—	+	—									
Johnin 110	10- 3-39	—	S	—	+								
Johnin 110	10-17-39	+	—	+	+	+	+	+	S	+			
Johnin 3A-1	10-31-39	S	—	+	S	S	+	+	S		+		
Johnin 1A	11-14-39	+	—	+	+	+	+	+	+		+	+	
Johnin 9A	1- 3-40	—	+	S	+	S	+	+	+		+	+	
Johnin 4F	2-13-40	—	S	+	+	+	+	+	S		+	+	
Destroyed	2-20-40												
P. M. lesions		—	—	—	+	—	+	+	+	+	+	+	+
Acid-fast smears		—	—	—	+	+	+	—	+	+	+	+	—

*Hatched 5-3-39. — = Negative.
+ = Positive. P.M. = Postmortem.
S = Slight. †Postmortem 8-12-39.

of johnin intradermically in the wattle of each chicken for each test. The variation in the intervals between retests, the number of retests and the test and postmortem findings of experiments with positive results are shown in tables II to VI.

The chickens which were inoculated as 5- and 10-day embryos with 0.01 Gm. of *M. paratuberculosis* suspended in 0.1 cc. of mineral oil were intradermically tested in the right wattle at the age of 3 months. Two of them gave slightly positive reactions. Monthly retests through the eighth month failed to produce additional reactions. Neither macroscopic lesions nor acid-fast organisms were observed on postmortem examination.

The chickens which were inoculated as 5- and 10-day embryos with 0.02 Gm. of *M. paratuberculosis* suspended in 0.2 cc. of mineral oil (tables II and III) showed an increased percentage of positive reactions, but a lack of uniformity between the first test and succeeding retests.

A group of chickens 1 day old and a similar group 1 month old were inoculated with 0.02 Gm. of *M. paratuberculosis* suspended in 0.2 cc. of mineral oil. The number tested, the intervals at which the tests were made and the postmortem findings are shown in tables IV and V.

In these experiments the second test two months after inoculation was usually positive and in several instances the reaction was more pronounced than the response elicited by the initial test, which was made one month after inoculation. Although some variation was observed in the response to retests, a much greater uniformity and stability of reaction were shown in these experiments than had been noted in either rabbits or guinea pigs.

In an effort to determine the effect of an increase in the quantity of organisms inoculated on the degree and stability of the resulting sensitivity, an additional group of chickens was inoculated. Table VI shows, with the exception of one suspicious reaction on each of three birds, that the results were positive on all tests and retests. This remarkable uniformity of testing results is the basis for the development of a method of evaluation and

TABLE VI—Results of Johnin Test and Postmortem Examinations on Chickens Inoculated as One-Month-Old Chickens* with 0.5 cc. of Mineral Oil Suspension of *M. Paratuberculosis*

| Test Material | Date | Bird Number | | |

standardization of johnin which the authors present in part II of this paper.

SUMMARY

Mycobacterium paratuberculosis suspended in mineral oil failed to produce a satisfactory sensitization in rabbits when injected either subcutaneously or intraperitoneally. Suspended in mineral oil, this organism failed to produce a uniform sensitization in guinea pigs when injected intraperitoneally. When suspended in mineral oil and injected subcutaneously in guinea pigs, it produced a satisfactory initial sensitization only.

Five- and 10-day-old chick embryos and day- and month-old chickens inoculated with 0.01 and 0.02 Gm. of *M. paratuberculosis* suspended in mineral oil failed to produce a uniform sensitization. Five-hundredths Gm. of the organism suspended in 0.5 Gm. of mineral oil inoculated intraperitoneally into month-old chickens produced a uniform and lasting sensitization.

II. A Proposed Method of Testing the Potencies of Johnins

To facilitate our attempts to develop a satisfactory intradermic johnin for the detection of Johne's disease, it was considered necessary to devise a reliable method for johnin potency determination.

In order to ascertain which of several species of small animals usually employed in laboratory work would best serve as test animals for the investigation of the relative potencies of different johnins, studies were carried out with rabbits, guinea pigs and chickens, as reported in part I of this paper. During the course of these studies it was found difficult to sensitize rabbits and the sensitivity produced in guinea pigs was transient or lacked uniformity. It has been shown also (unpublished data) that a reproducible end-point reaction with the dilutions employed is obtained when tests of the same lots of johnin are applied to successive groups of chickens sensitized in an identical manner. However, different strains of *M. paratuberculosis* produce varying degrees of sensitivity in chickens. A reliable method for evaluating the potencies of johnins by the use of sensitized chickens is, therefore, proposed.

This paper describes the technic used in sensitizing chickens to *M. paratuberculosis* and gives the method employed in testing johnin and recording results of such tests.

MATERIALS AND METHODS

The johnins used in these experiments were made from synthetic culture mediums on which *M. paratuberculosis* had grown satisfactorily. The method employed to produce intradermic johnin from mature cultures of the organism was similar to that used by Dorset and Henley[1] to produce intradermic tuberculin from mature cultures of *M. tuberculosis*.

Because of the universal use of the term "O.T." to designate concentrations of tuberculin, a similar term "O.J." is used in this paper to designate concentration of johnin. That is, a johnin of O.J. concentration is one prepared by concentrating a culture filtrate to 10 per cent of the basal volume of the medium. In practice, the filtrate from a culture planted originally on 100 cc. of medium was evaporated to 9 cc., and 1 cc. of a 5 per cent phenol solution was added.

The chickens used were White Leghorn cockerels purchased from an accredited hatchery at one day of age. White Leghorns were chosen because the large and rapidly developing wattles, which are characteristic of the males of this breed, are convenient for the intradermic injection of johnin and for observations of reactions. As soon as the wattles were developed sufficiently, that is, when the chickens were from 4 to 8 weeks old, johnin tests were made to determine whether a sensitivity existed in any of the birds. Only those cockerels which showed no reactions at the end of 48 hours were used.

Material for sensitizing chickens was prepared as follows: Cultures of *M. paratuberculosis* were planted on the surface of liquid mediums and, after a month, the resulting organisms were separated from the mediums by filtering through sterile Seitz filters. After all of the liquid had been drawn through the filter, a slight vacuum was maintained for about ten minutes to dry the organisms partially. This facilitated their suspension in oil. The partially dried mass was weighed and placed in a sterile, covered mortar. For each gram of organisms 10 cc. of sterile mineral oil was added slowly with constant stirring. This suspension was then placed in test tubes and shaken in a shaking machine for one hour.

Each bird to be sensitized was injected intraperitoneally with 0.5 cc. of this suspension. One month after inoculation and at two-week intervals thereafter until a marked and relatively uniform sensitivity appeared, two chickens

TABLE VII—Work Chart for Potency Test, Johnin 6-G

Bird No.	Dilutions*	24 Hrs.	48 Hrs.	72 Hrs.	Bird No.	Dilutions*	24 Hrs.	48 Hrs.	72 Hrs.
675	1:4	2+	4+	4+	685	1:400	2+	2+	2+
676	1:4	3+	4+	4+	686	1:400	2+	3+	4+
677	1:4	2+	4+	3+	687	1:400	2+	4+	4+
678	1:4	2+	4+	3+	688	1:400	2+	4+	3+
679	1:4	3+	4+	4+	689	1:400	1+	2+	1+
Total		12	20	18	Total		9	15	14
Average		2.4	4.0	3.6	Average		1.8	3.0	2.8
680	1:40	2+	3+	4+	690	1:4000	1+	1+	—
681	1:40	2+	4+	4+	691	1:4000	1+	2+	2+
682	1:40	Died 7-12-40			693	1:4000	1+	1+	—
683	1:40	3+	3+	2+	763	1:4000	½+	½+	—
684	1:40	3+	4+	4+	764	1:4000	2+	½+	—
Total		10†	14	14	Total		5.5	5	2
Average		2.4	3.5	3.5	Average		1.1	1.0	0.5

*Expressed in O.J. units.
†Four birds only.
½+ = Small, circumscribed swelling at point of injection.
1+ = Edema of 50 per cent of injected wattle.
2+ = Edema of the entire wattle.
3+ = Entire injected wattle and 50 per cent of opposite wattle edematous.
4+ = Both wattles edematous.

were tested intradermically in the right wattle with johnin prepared from the same strain of *M. paratuberculosis* that was used to sensitize the birds. Within six to eight weeks after inoculation all chickens had developed maximum sensitivity and were ready for use in determining potency titers.

To determine the relative potencies of various lots of johnin prepared from different strains of *M. paratuberculosis*, four different dilutions of each johnin to be tested were made by the following procedure:

Intradermic johnin, which contains only 25 per cent of O.J. and is, therefore, already a 1:4 dilution, was used as the basis for higher dilutions. Then a 1:10 dilution of the intradermic johnin would be equivalent to a 1:40 dilution of O.J., or 2.5 per cent O.J. By the same precept the 1:400 dilution of O.J., which was a 1:10 dilution of the previous one, contains 0.25 per cent O.J.; 1:4,000 contains 0.025 per cent O.J.; and 1:6,000 contains 0.01875 per cent O.J. The diluent used was 0.5 per cent phenol in 0.85 per cent sodium chloride solution. Each dilution was tested on five chickens previously sensitized with the same strain of the organism which was used in making the johnin. One-tenth cc. injections were made intradermically in the right wattle. At two-week intervals similar dilutions of other lots of johnin were subsequently injected alternately into right and left wattles.

The following system of recording reactions numerically was arbitrarily adopted. A small, circumscribed swelling at the point of injection was recorded as ½ plus; edema involving 50 per cent of the injected wattle, 1 plus; edema involving the entire injected wattle, 2 plus; edema of the entire injected wattle and 50 per cent of the opposite wattle, 3 plus; and edema involving the entire mass of both wattles, 4 plus. Examples of different degrees of reactions are shown in table VII.

The average index of reaction for a given dilution of a lot was determined by adding the individual indexes of all of the birds injected with that dilution and dividing the sum by the number of birds. Average indexes for each dilution of each lot were obtained 24, 48 and 72 hours following injections. Since the 48-hour indexes were in most instances the highest, they were used in evaluating the potencies of the different lots of johnin.

Discussion

The authors are proposing that the term "potency titer" be applied to the highest dilution at which a johnin will elicit a satisfactory reaction in birds sensitized to *M. paratuberculosis*. An average reaction index of 1 was considered the minimum which indicated a satisfactory response.

Table VIII sets forth the system employed for the purpose of assigning "potency

TABLE VIII—Potency Titer Interpretation (Based upon Average Reactions of Five Sensitized Birds per Dilution)

Dilutions*					Potency Titer
1:4	1:40	1:400	1:4,000	1:6,000	
—	—	—	—	—	<1:4
+	—	—	—	—	>1:4 <1:40
+	+	—	—	—	>1:40 >1:400
+	+	+	—	—	>1:400 <1:4,000
+	+	+	+	—	>1:4,000 <1:6,000
+	+	+	+	+	>1:6,000

*All dilutions in terms of O.J.
< = Less than.
> = Greater than.
— = Average reaction of five sensitized birds 0.9 or less.
+ = Average reaction of five sensitized birds 1 or greater.

titers" to different lots of johnin. A positive (+) in any dilution of johnin employed indicates that the average reaction of the five birds tested with the dilution is one (1) or greater. A negative (—) in any dilution of johnin indicates that the average reaction of the five birds tested with the dilution is nine-tenths (0.9) or less. For example, the potency titer of one lot of johnin is computed as follows:

Lot No.	Dilution	Average 48-Hr. Reading for 5 Birds	Potency Titer
6J	1:4	4.0 +	
	1:40	3.4 +	
	1:400	2.25+	
	1:4,000	1.0 +	(Greater than)
	1:6,000	0.62—	(Less than)

The potency titer, then, may have been higher than the highest dilution which gave an average index of 1 or more, but lower than the next higher dilution employed in the series.

Whether or not it will be possible to detect variations in potency within a more limited range of dilutions than those employed has not been sufficiently tested to state the exact limitations of this test. Investigations are now in progress to determine this point.

SUMMARY

The proposed term "potency titer" and "O. J." are defined.

A technic for measuring the "potency titer" of johnin is described. It consists of sensitizing chickens to johnin by intraperitoneal injections of mineral oil suspensions of *Mycobacterium paratuberculosis* and determining the highest dilution of johnin under test which will elicit satisfactory reactions when injected intradermically in such chickens.

Some Observations on the Pathology of Pneumonia in the Food-Producing Animals*

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PNEUMONIA AS A distinct entity has been recognized since the inception of bacteriology. The young of the three main food-producing mammals, namely, cattle, sheep and swine, suffer severe yearly losses from this disease. This investigation was initiated in order to secure further information on the pathology of this disease among the three types of animals mentioned.

Very little work has been published on the cellular pathology of pneumonia among the lower animals. Thorp and Hallman[1] described the pathology of bronchopneumonia in calves and associated beta streptococci. Thorp and Tanner[2] reported on a bacteriologic study of the aerobic flora of pneumonic lungs in swine. Streptococci were isolated more frequently than Pasteurella. Spray[3] reported Pasteurella to be most frequently associated with sheep pneumonia. Newsom and Cross[4] and Rosenbusch and Merchant[5] published work on the bacteriology of Pasteurella isolated from ovine pneumonic lungs.

BACTERIOLOGIC STUDIES

The lungs were secured from animals submitted to the Pathology Department for diagnosis.

The surface of the lung to be cultured was seared with a red hot spatula. Two small squares of tissue were removed with a sterile forceps and scissors. A blood (5 per cent sheep) agar plate was streaked with one piece of the tissue and incubated for 24 hours or longer at 37 C. The other piece was placed in tryptose broth and incubated 8 to 10 hours at 37 C. Following incubation blood-agar plates were then streaked and incubated for 24 hours at 37 C. The cultures were then studied bacteriologically. The bacteria were identified on the basis of colony morphology, gram stain, motility and the use of the following mediums: litmus milk, gelatin, tryptophane, nitrate, glucose, lactose, maltose, mannite, sucrose and salicin. The mediums and tests were prepared and executed according to Stafseth's manual[6] which gives standard procedures.

The streptococci were studied serologically by the methods of Lancefield[7] and Fuller.[8] According to these procedures the beta streptococci were classified as belonging to Lancefield's group C.

Table I records the results obtained after studying the aerobic flora isolated from four bovine lungs and two throat swabs. Beta streptococci belonging to Lancefield's group C were isolated in each instance.

Table II records the results of culturing the lungs of nine sheep and lambs. *Pasteurella oviseptica* was isolated from eight of the nine lungs examined. Beta streptococci (Lancefield's group C) and Spray's diplococcus were isolated three and four times, respectively.

Table III records the results of bacteri-

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†Resigned September 1, 1939.

[1]Thorp, W. T. S., and Hallman, E. T.: Pathology of calf pneumonia. J.A.V.M.A., xciv (1939), pp. 365-371.

[2]Thorp, F. Jr., and Tanner, F. W.: A bacteriological study of the aerobic flora occurring in pneumonic lungs of swine. J.A.V.M.A., xcvi (1940), pp. 149-160.

[3]Spray, R. S.: Bacteriologic study of pneumonia in sheep. J. Inf. Dis., xxxiii (1923), pp. 97-112.

[4]Newsom, I. E., and Cross, F.: Some bipolar organisms found in pneumonia in sheep. J.A.V.M.A., lxxx (1932), pp. 711-719.

[5]Rosenbusch, C. T., and Merchant, I. A.: A study of the hemorrhagic septicemia Pasteurella. J. Bact., xxxvii (1939), pp. 69-89.

[6]Stafseth, H. J.: Laboratory guide in pathogenic bacteriology. (Bact. 304f, revised Jan. 1940. Mimeograph Dept., Michigan State College, East Lansing, Mich.)

[7]Lancefield, R. C.: A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med., lvii (1933), pp. 571-595.

[8]Fuller, A. T.: The formamide method for the extraction of polysaccharides from hemolytic streptococci. British J. Exp. Path., xix (1938), pp. 130-139.

TABLE I—Microörganisms Isolated from Four Bovine Lungs and Two Throat Swabs

Microorganisms	Cases					
	1	2	3	4	5*	6*
Beta streptococcus	+	+	+	+	+	+
Staph. aureus		+	+	+		
Staph. albus	+					
Coliform	+	+		+		

*Throat swabs.

TABLE II—Microörganisms Isolated from Nine Pneumonic Ovine Lungs

Microorganisms	Cases								
	1	2	3	4	5	6	7	8	9
P. oviseptica	+	+	+	+	+	+	+	+	
Beta streptococcus		+	+	+					
Spray diplococcus						+	+	+	+
Staph. aureus	+		+						
Staph. albus			+						
Coliform	+	+	+						
Neisseria catarrhalis-like							+		+

TABLE III—Microörganisms Isolated from Five Pneumonic Porcine Lungs

Microorganisms	Cases				
	1	2	3	4	5
P. suilla	+	+		+	+
Beta streptococcus		+	+	+	+
Corynebacterium					+
Staph. aureus					+
Staph. albus			+		
Coliform		+			
S. suipestifer			+		

ologic examinations on five of the porcine lungs studied. *P. suilla* and beta streptococci (Lancefield's group C) were each isolated from four of the lungs, three times in combination from the same lungs. The other five microörganisms were isolated from three of the five lungs.

Gross Pathology

In the food-producing animals the gross pathology of bronchopneumonia was similar with few exceptions. The bronchopneumonia was bilateral in distribution, involving the apical, cardiac, intermediate and usually the anterior part of the diaphragmatic lobes (figs. 1 and 2). The lesions were usually patchy, showing areas of red and gray consolidation. Many times the zones between the consolidated areas and the normal lung tissue were markedly congested. Atelectasis and emphysema often were observed. The latter condition appeared to be more common in the bovine species. In a few cases some areas of induration were noted. Necrosis and abscesses sometimes complicated the pneumonias, especially the porcine.

When the trachea and bronchi were opened, a grayish white, tenacious exudate was sometimes present. The pleura and interstitial tissues were sometimes thickened with an edematous and fibrinous exudate. A few fibrinous and fibrotic adhesions were present.

Technic

The histopathologic technic was the same as that described by Thorp and Hallman[1] in 1939. In addition, Heidenhain's aniline blue stain, and Verhoeff's elastic tissue stain were used for differential diagnosis (Mallory[9]).

Histopathology

The histopathology was studied on 31 cases of bronchopneumonia, 17 of which were acute, 13 subacute and 1 chronic. The process appeared to begin in the smaller bronchioles and adjacent alveoli, making the pneumonia appear patchy in distribution (fig. 3). In the acute cases the exudate consisted primarily of polymorphonuclear leucocytes, some mononuclear phagocytes and occasionally putrid exudate. The bronchial epithelium many times was swollen, vacuolated, desquamated and occasionally necrotic (fig. 4). The lamina propria in a few cases was inflamed, showing some congestion, and the exudate consisted of neutrophiles, lymphocytes and some fibrin. The peribronchial areas were

[9]Mallory, F. B.: Pathological technic (W. B. Saunders Co., Philadelphia, 1938), pp. 154, 170.

occasionally infiltrated with lymphocytes, neutrophiles and some mononuclear phagocytes (fig. 5). In a few instances the entire wall was necrotic.

The alveoli adjacent to the respiratory bronchioles showed an exudate consisting primarily of polymorphonuclear leucocytes

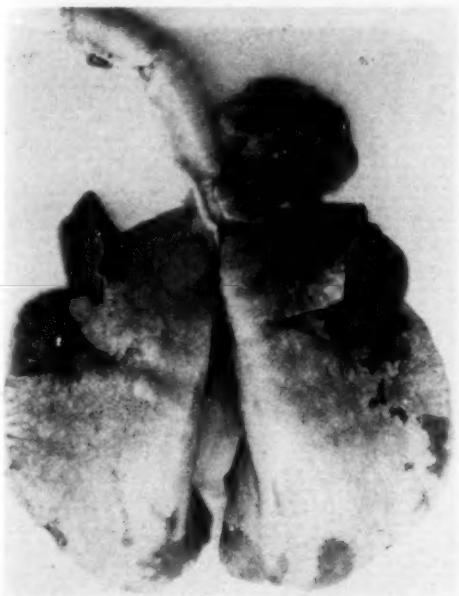

Fig. 1. Bronchopneumonia, sheep's lung, dorsal view (approximately one-fourth natural size).

together with a few monocytes. In one or two cases the monocytes appeared to be the primary cell. In the alveoli beyond the patchy areas various stages of inflammation were noted. The exudate was serous, fibrinous, lymphocytic and hemorrhagic, and contained some polymorphonuclear leucocytes and mononuclear phagocytes (fig. 6). The dominant cells in these areas were mononuclear phagocytes (fig. 7). The alveolar walls often were thickened by a marked congestion and increase in the neutrophiles, mononuclear phagocytes and lymphocytes (fig. 8). In a few instances the alveolar walls appeared thickened because of the swollen endothelial and septal cells. Due to the accumulation of exudate in the bronchioles, atelectasis was commonly present along with some emphysema (figs. 9 and 10). In a few areas the alveolar walls were partially to completely destroyed due to necrosis.

In a few cases the large bronchi showed inflammatory changes. The lumens contained an exudate of polymorphonuclear leucocytes which was occasionally putrid. The epithelium was sometimes swollen and vacuolated, and showed some desquamation and necrosis. The lamina propria occasionally showed congestion and an infiltration of polymorphonuclear leucocytes and lymphocytes (fig. 11). The peribronchial areas sometimes showed focal accumulations of lymphocytes simulating a lymph follicle. Other changes observed were congestion and increased numbers of polymorphonuclear leucocytes.

The interstitial tissues often were thickened due to edema, fibrinous exudate, hemorrhagic exudate and cellular exudate consisting of neutrophiles and lymphocytes along with a few macrophages. The lymph

Fig. 2. Ventral view of lung shown in figure 1.

vessels often were distended with fluid and cellular exudate.

The pleura often showed varying degrees of thickness due to increased amounts of a serofibrinous exudate (fig. 12). Some polymorphonuclear leucocytes and lymphocytes were present. The pleural blood vessels of-

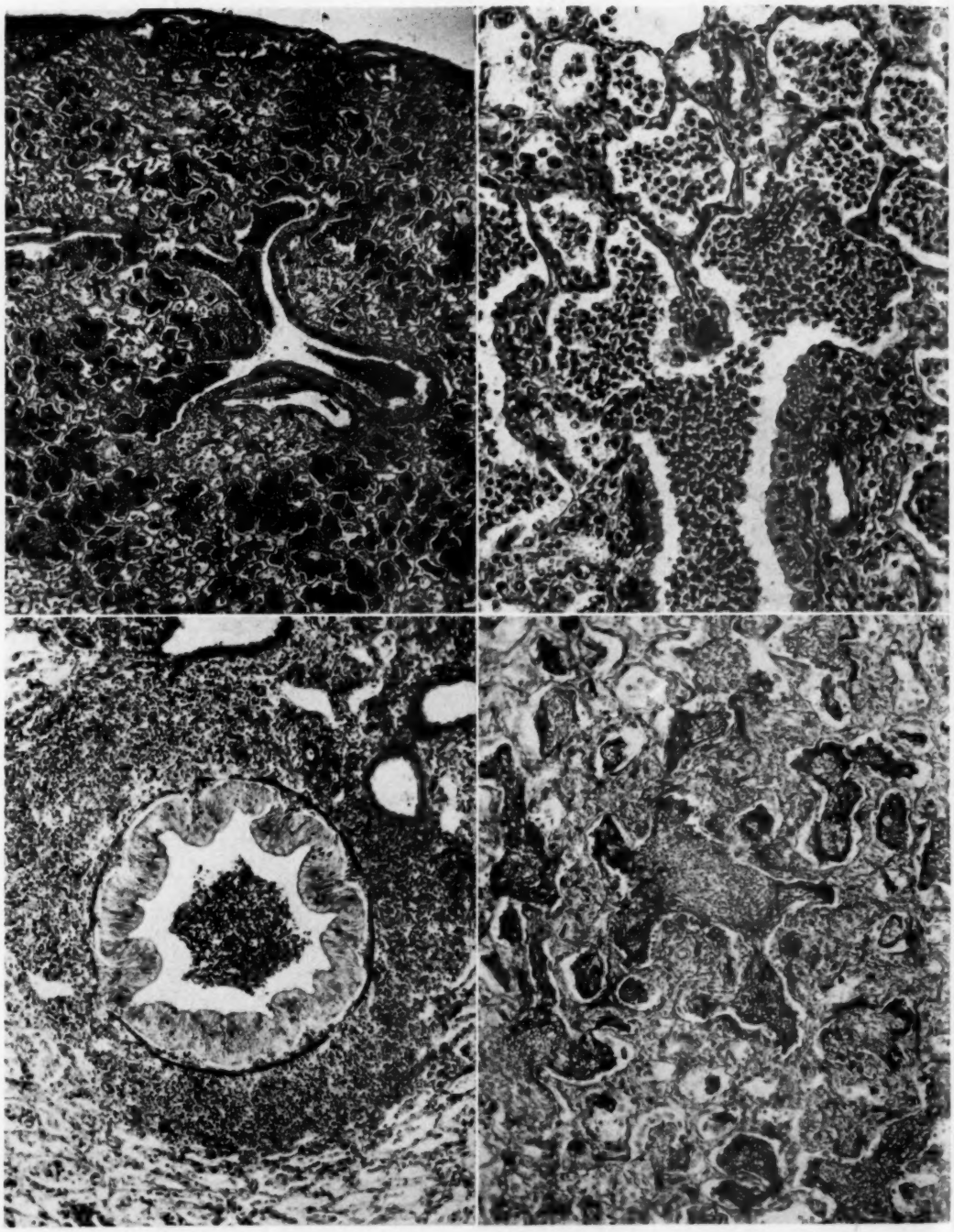

Fig. 3 (upper left). Patchy pneumonia with exudate in respiratory bronchiole and surrounding alveoli (x 200).

Fig. 4 (upper right). Exudate of polymorphonuclear leucocytes in a respiratory bronchiole and surrounding alveoli. The epithelium of the respiratory bronchiole is swollen (x 225).

Fig. 5 (lower left). Peribronchial infiltration with lymphocytes and macrophages (x 98).

Fig. 6 (lower right). Fibrinous exudate in alveoli (x 98).

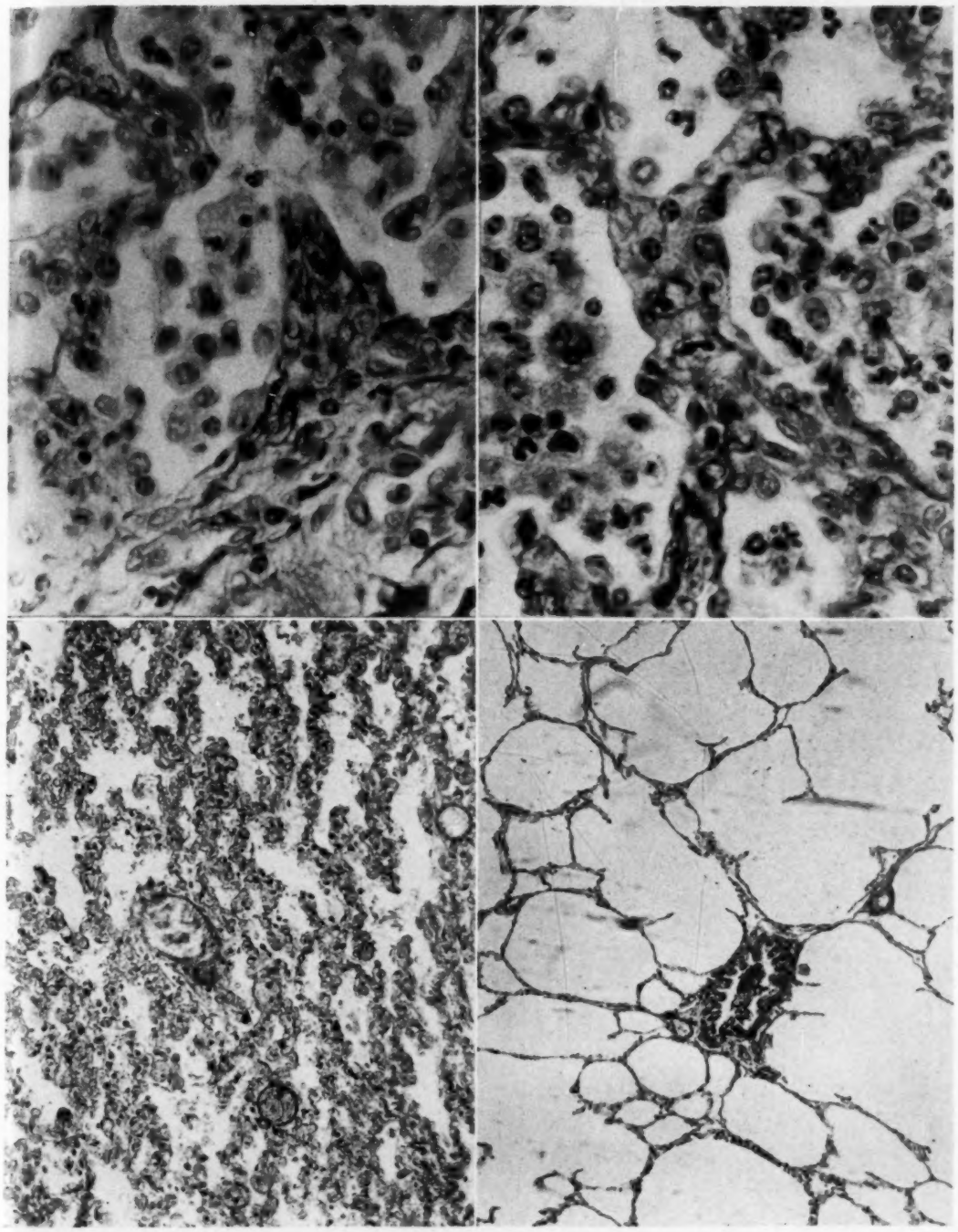

Fig. 7 (upper left). Alveoli containing macrophages (x 520).
Fig. 8 (upper right). Thickening of the alveolar walls due to congestion and accumulations of polymorphonuclear leucocytes, macrophages and lymphocytes (x 750).
Fig. 9 (lower left). Atelectasis and congestion (x 135).
Fig. 10 (lower right). Marked emphysema (x 131).

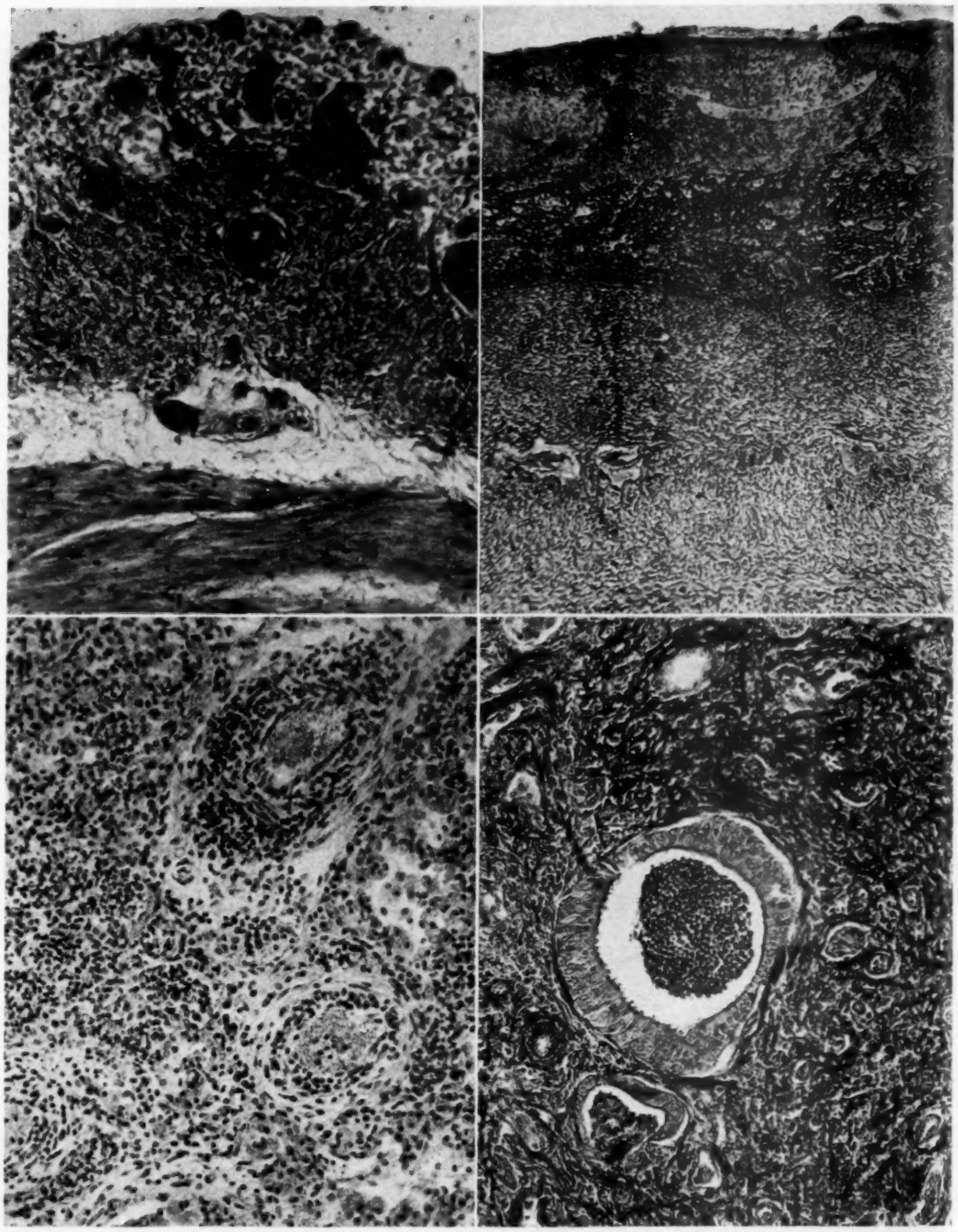

Fig. 11 (upper left). Portion of a large bronchus, showing congestion, inflammatory cells in the lamina propria and loss of epithelium (x 135).

Fig. 12 (upper right). Subacute pleuritis characterized by marked serofibrinous exudate at surface of pleura and proliferation of fibroblasts and fibrosis adjacent to the alveoli (x 56).

Fig. 13 (lower left). Leucocytic infiltration of the media and adventitia of one artery and the intima of the other (x 150).

Fig. 14 (lower right). Peribronchial fibrosis and metaplasia of bronchial epithelium (x 124).

ten showed varying degrees of congestion. The lymphatic vessels showed changes similar to those described for the interstitial tissues. The areas at the junctions of the pleura and the interstitial tissues revealed the most marked changes.

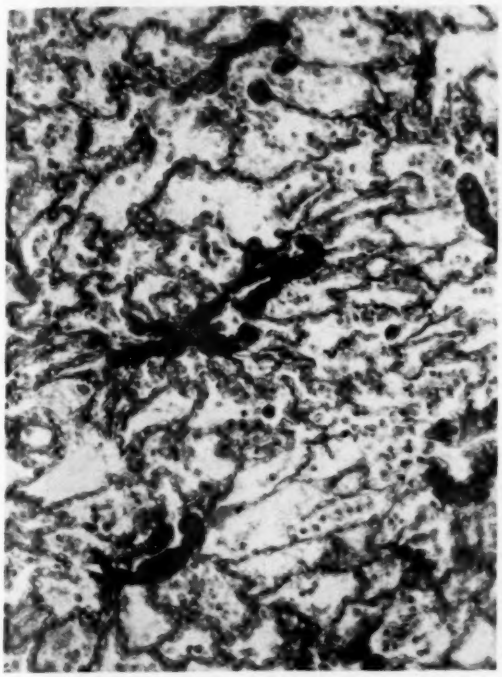

Fig. 15. Hyperplasia of smooth muscle of walls of alveolar ducts (x 98).

Some of the arteries and veins showed inflammatory changes involving one or all of the three walls (fig. 13). The most commonly involved layer was the adventitia, with infiltrations of lymphocytes, polymorphonuclear leucocytes, serous exudate and hemorrhagic exudate. Occasionally the media and intima revealed increased numbers of these inflammatory cells. Thrombosis of the arteries or veins was observed in four cases.

In the subacute bronchopneumonia cases many lesions similar to those described for the acute were present. Often the subacute cases had many of the acute reactions, and in addition showed proliferation of fibroblasts and fibrosis in the various structures (fig. 14). In a few areas showing the most chronicity some of the alveoli appeared to be lined with cells resembling cuboidal epithelium. Hyperplasia of the smooth muscle was observed in several cases (fig. 15). Metaplasia of the bronchial epithelium was seen several times. In two cases of pleuritis the mesothelium appeared to be cuboidal.

The one chronic case showed a generalized fibrosis of the lung and pleura.

The histopathology of bronchopneumonia in the food-producing animals was quite similar in most cases. In sheep and hog lungs there was hyperplasia of the smooth muscle. They both showed more necrotic foci than were observed in the bovine lungs. Some of these necrotic areas resembled infarcts and in one or two instances thrombi were noted in the blood vessels of the areas.

SUMMARY

The pathology was studied on 31 cases of bronchopneumonia—nine bovine, twelve ovine and ten porcine. Beta streptococci (Lancefield's group C) were isolated from four of the bovine lungs. In the ovine cases *Pasteurella oviseptica* was isolated eight times, beta streptococci three times, and Spray's diplococci four times. In the porcine cases *P. suilla* and beta streptococci were each isolated four times.

The gross pathology usually was confined to the apical and cardiac lobes, but it sometimes involved the diaphragmatic and intermediate lobes.

The bronchopneumonia appeared to begin as a bronchiolitis which soon spread to the adjacent alveoli, giving it a patchy appearance. The cellular exudate usually was dominated by polymorphonuclear leucocytes, but in two cases the dominant cells were mononuclear phagocytes. The interstitial tissues, pleura and blood vessels occasionally showed various stages of inflammation.

In the 13 subacute cases and one chronic case there was proliferation of fibroblasts and some fibrosis.

Studies on the Allergic and Antigenic Activity of Sonic Filtrate of Brucella Abortus*

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THE PRACTICABILITY OF an allergic skin test to establish Brucella infection has been investigated by various workers, and different agents have been used for that purpose.

REVIEW OF LITERATURE

Reichel and Harkins[1] used a heat-killed suspension of *Brucella abortus*. The dose they injected intracutaneously into the caudal fold of cattle contained 500 million bacteria. They found that animals negative to the agglutinative test did not react to the intracutaneous injection. However, only a small percentage of serologically positive animals gave a positive intracutaneous reaction. Fleishner and Meyer[2] found that injections of dead Brucella into guinea pigs stimulated the production of agglutinins but did not induce skin sensitivity. On the other hand, infection of guinea pigs with live *Br. abortus* always led to cutaneous hypersensitivity to Brucella protein. Stafseth[3] applied the intracutaneous test to guinea pigs infected with *Br. abortus*. He obtained better results with the antigen of Reichel and Harkins[1] than with the alcohol-ether extract of Fleishner and Meyer.[2] The injection of dead culture had no influence upon the reaction.

Goldstein,[4] in comparing the results of cutaneous reactions in man to whole organisms killed by heat, and to an ether-extracted, fat-free antigen, came to the conclusion that the latter was preferable because it did not evoke systemic reactions and produced a lesser degree of ulceration at the site of injection. Cordy[5] infected guinea pigs with *Br. abortus* and tested their skin sensitivity with a heat-killed suspension, a crude nucleoprotein fraction and a broth culture filtrate of Brucella. He stated that none of the agglutination-positive, artificially infected animals showed negative skin reactions and none of the controls showed positive skin reactions. Pennell and Huddleson[6] prepared "endo-antigen" by digesting ground Brucella organisms with trypsin. This preparation elicited specific skin reactions in sensitized animals. Stahl, Pennell and Huddleson[7] used protein-nucleate of Brucella to determine its allergic activity on sensitized rabbits. Upon separation of the protein from the nucleic acid they found that practically all of the allergic activity lies in the protein, and that the nucleic acid portion of the protein-nucleate plays no part in producing an allergic reaction.

Following a method for the preparation of P.P.D. (purified protein derivative from tuberculin), Morales-Otero and Gonzales[8] obtained a protein product from *Br. abortus* which elicited skin reactions in infected guinea pigs. In further studies with this purified protein on human beings[9] they found that no correlation existed between cutaneous allergy and serologic reactions. Huddleson,[10] comparing skin reactions in human beings to intracutaneous injections of brucellergen with the agglutinative titers of individuals, came to the following conclusion: "If the agglutination test alone had been used to determine past and present infection it would have failed to detect 86.9 per cent of the 845 individuals studied."

In discussing bacterial autolysates, Jordan and Falk[11] stated: "In working with materials as vulnerable to chemical insult as are biologically active constituents of any cells, it is

*From the School of Veterinary Medicine and the School of Animal Pathology, University of Pennsylvania. These studies were supported by funds from the Pennsylvania Department of Agriculture, Pennsylvania Department of Health and the Bureau of Animal Industry, U. S. Department of Agriculture.

[1]Reichel, J., and Harkins, M. J.: The diagnosis of infectious abortion of cattle (Bang's disease) with special reference to the intradermal test. J.A.V.M.A., I (1917), p. 846.

[2]Fleishner, E. C., and Meyer, K. F.: The bearing of cutaneous hypersensitiveness on the pathogenicity of the *Bacillus abortus bovinus*. Am. J. Dis. Child., xvi (1918), p. 268.

[3]Stafseth, H. J.: Studies in infectious abortion. Michigan Agr. Exp. Sta. Tech. Bul. 49 (1920).

[4]Goldstein, J. D.: Cutaneous reactions in the diagnosis of undulant fever. J. Clin. Investig., xiii (1934), p. 209.

[5]Cordy, D. R.: A study of the intradermal test for brucellosis in guinea pigs. Ann. Rpt. New York State Vet. Coll. at Cornell Univ. (1937-38).

[6]Pennell, R. B., and Huddleson, I. F.: Chemical constitution and biological properties of the endo-antigen of the Brucella groups of microörganisms. Michigan Agr. Exp. Sta. Tech. Bul. 156 (1937).

[7]Stahl, W. H., Pennell, R. B., and Huddleson, I. F.: A study of the protein-nucleates of the species of the genus Brucella. Michigan Agr. Exp. Sta. Tech. Bul. 168 (1939).

[8]Morales-Otero, P., and Gonzales, L. M.: Purified protein antigen from Brucella. Proc. Soc. Exp. Biol. & Med., xxxviii (1938), p. 703.

[9]*Ibid.*: Allergy in Brucella infections. Proc. Soc. Exp. Biol. & Med., xl (1939), p. 100.

[10]Huddleson, I. F.: Brucellosis in man and animals (The Commonwealth Fund, New York, 1939), p. 226.

[11]Jordan, E. O., and Falk, I. S.: The newer knowledge of bacteriology and immunology (2nd ed., University of Chicago Press, Chicago, 1929), p. 728.

obvious that injury to antigenic function may easily occur." The authors pointed out that though qualitatively there is no difference between the nucleoprotein prepared from bacteria by chemical manipulation and their autolysates, the latter represent a more potent, less denatured form of nucleoprotein because of the more gentle method of its liberation.

It has been shown by Chambers and Gaines,[12] Harvey and Loomis[13] and Chambers and Weil[14] that sonically killed bacteria are disintegrated. Chambers and Flosdorf[15] demonstrated that some heat-labile proteins are not denatured by sonic treatment. By using this method Chambers and Flosdorf[16] further succeeded in extracting from *Eberthella typhi* the "Vi" antigen which had not been separated by any other means of bacterial destruction. Mudd *et al.*[17, 18] subjected streptococci to sonic vibration, and found that the bacterial extract thus obtained absorbed antibodies from immune serums.

MATERIALS AND METHODS

In the present study the sonic method of disintegrating Brucella was used. Rabbits and guinea pigs were employed in the experiments to ascertain the allergic activity of sonic extract of Brucella. In addition agglutinative, precipitative and opsonocytophagic tests were carried out.

Strain 53 of *Br. abortus* was used. It had been isolated from the stomach contents of an aborted fetus and was supplied to us by Dr. M. F. Barnes of the Pennsylvania Bureau of Animal Industry. Large quantities of culture were grown on tryptose agar in flat, 32-oz. bottles for 72 hours at 37 C. At the end of the incubation period about 10 cc. of 0.85 per cent NaCl was added to each bottle and left for 10 to 15 minutes to wash the growth off the surface of the medium. The saline suspension of cells was removed, centrifuged at high speed for about one hour and the supernatant fluid was poured off. Then the sediment was resuspended in sterile saline to the original volume and transferred to the treatment vessel of the magnetostriction oscillator described by Chambers and Flosdorf.[16] The bacterial suspensions were exposed for one to two hours to vibration of audible frequency at 9,000 cycles per second. Subsequently the contents were removed from the glass vessel and centrifuged at high speed to remove any intact cells and débris. The supernatant fluid was then diluted with an equal quantity of sterile saline and passed through a Seitz filter. The filtrate was clear and was checked for sterility in each instance by transferring 0.5 cc. to a tube of tryptose broth and to a tryptose-agar plate. A macro-Kjeldahl determination of protein was carried out on each lot of sonic filtrate, and the concentration of protein varied in different lots from 1 to 3 mg. per cubic centimeter. For the intracutaneous and precipitative tests progressive dilutions of the filtrate were prepared with sterile saline to correspond to the desired dilutions of protein.

Agglutinative Tests.—The antigen used in the agglutinative tests was a suspension of *Br. abortus* strain 53 in 0.85 per cent NaCl with the addition of 0.5 per cent phenol crystals. The suspension was diluted with phenol saline solution to compare with tube no. 1 of the McFarland[19] nephelometer.

Precipitative Tests.—In carrying out the precipitative tests, 0.1 cc. of antigen in serial dilution was mixed with 0.1 cc. of serum. The tubes were incubated for two hours at 37 C., allowed to stand in the refrigerator overnight, then centrifuged at high speed and examined for the presence of a sediment. In the control tubes 0.1 cc. of serum on one hand, and 0.1 cc. of antigen on the other, were mixed with an equal quantity of saline. The antigen dilutions were calculated according to the protein content of the filtrate.

Opsonocytophagic Tests.—These were conducted with citrated whole blood and *Br. abortus* according to the technic described by Huddleson.[10] One cc. of blood was added to 0.05 cc. of a 16 per cent solution of sodium citrate in 0.85 per cent NaCl, resulting in a final dilution of 0.8 per cent of sodium citrate in the blood. One-tenth cc. of citrated blood was mixed in a small test tube with 0.1 cc. of a 48-hour culture of *Br. abortus* which had been suspended in saline and adjusted in density to compare with tube 16 of the McFarland nephelometer. The blood-Brucella mixture was shaken, placed in a water bath at 37 C. for 30 minutes and shaken again. Then smears were made and stained with a preparation of toluidine blue ac-

[12]Chambers, L. A., and Gaines, N.: Some effects of intense audible sound on living organisms and cells. J. Cell & Comp. Physiol., i (1932), p. 451.

[13]Harvey, E. N., and Loomis, A. L.: The destruction of luminous bacteria by high-frequency sound waves. J. Bact., xvii (1929), p. 373.

[14]Chambers, L. A., and Weil, A. J.: Immunological properties of sonic extract of pneumococci. Proc. Soc. Exp. Biol. & Med., xxxviii (1938), p. 924.

[15]Chambers, L. A., and Flosdorf, E. W.: The denaturation of proteins by sound waves of audible frequencies. J. Biol. Chem., cxiv (1936), p. 75.

[16]*Ibid.*: Sonic extraction of labile bacterial constituents. Proc. Soc. Exp. Biol. & Med., xxxiv (1936), p. 631.

[17]Mudd, S., Czarnetzky, E. J., Lackman, D., and Pettit, H.: The antigenic structure of hemolytic streptococci of Lancefield group A. J. Immunol., xxxiv (1938), p. 117.

[18]Mudd, S., Pettit, H., Lackman, D. B., and Morgan, I. M.: The antigenic structure of hemolytic streptococci of Lancefield group A. J. Immunol., xxxvi (1939), p. 381.

[19]McFarland, J.: The nephelometer. J.A.V.M.A., xlix (1907), p. 1176.

TABLE I—Skin Reactions in Rabbits in Response to Intracutaneous Injection of Sonic Filtrate

Rabbit Number		Allergic Skin Reactions After Injection of Brucella Abortus									
		48 Days					85 Days				
		Dilution of Protein in Filtrate					Dilution of Protein in Filtrate				
		1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:500	1:1,000	1:2,000	1:4,000	1:8,000
600	Injected	+3	+2	+2	+2	—	+2	+2	+2	+1	+2
601	with live	+3	+2	—	—	—	+3	+2	+2	+1	—
602	Brucella	+4	+3	+3	+3	+1	+4	+3	+3	+2	+1
603	intravenously	+3	+2	+2	+2	—	+3	+3	+2	+2	+1
604		+4	+3	+2	+2	+2	+3	+2	+2	+2	+1
606		+3	+2	+2	+2	+1	+3	+2	+2	+1	+1
607		+3	+2	+2	+2	+1	+3	+3	+2	+2	+1
608		+3	+3	+3	+3	+2	+4	+3	+3	+2	+2
609		+3	+2				+3	+3	+2	+2	+2
610	Injected	+3	+2	+1	—	—	+4	+3	+2	+2	—
611	with live	+3	+2	+1	—	—	*				
612	Brucella	—	—	—	—	—	—	—	—	—	—
613	intraperi-	+3	+3	+2	+1	—	+2	+2	+1	+1	—
614	toneally	+3	+2	—	—	—	+4	+3	+3	+3	+2
615		+3	+2	+1	—	—	*				
616		+3	+2	—	—	—	+4	+4	+3	+3	+2
617		—	—	—	—	—	+3	+2	+2	+2	+1
618		+4	+3	+2	+2	+1	+4	+4	+3	+3	+2
620	Injected with	+4	+3	—	—	—	*				
621	phenolized	—	—	—	—	—	—	—	—	—	—
622	culture intra-	+1	+1	—	—	—	*				
624	venously	—	—	—	—	—	—	—	—	—	—
625	Injected with	+4	+3	+3	+2	+1	+3	+3	+3	+2	+2
626	heated	+4	+3	+2	+2	+1	+2	+2	+2	+2	+1
627	culture	—	—	—	—	—	—	—	—	—	—
628	intravenously	+4	+3	+3	+3	+2	*				
629		—	—	—	—	—	+3	+2	+2	+2	+1
630	Noninjected	—	—	—	—	—	—	—	—	—	—
631	controls	—	—	—	—	—	+2	+2	+2	+1	+1
632		—	—	—	—	—	+2	+1	—	—	—
633		—	—	—	—	—	—	—	—	—	—
635		—	—	—	—	—	—	—	—	—	—
636		—	—	—	—	—	—	—	—	—	—
637		+2	+1	+1	—	—	—	—	—	—	—
638							—	—	—	—	—
639							—	—	—	—	—
640							—	—	—	—	—
641							—	—	—	—	—
642							—	—	—	—	—

*Died before the second test. – No reaction. +1, Erythema and edema up to 5 mm. in diameter. +2, Erythema and edema 5 to 10 mm. in diameter. +3, Erythema and edema 10 to 15 mm. in diameter. +4, Erythema and edema 15 to 20 mm. in diameter.

cording to a technic of Calmette, Négre and Boquet,[20] as cited by Huddleson.[19] Twenty-five neutrophiles were examined and the degree of phagocytosis was recorded.

Technic of Intracutaneous Tests.—Barium sulfide was used as a depilatory. One-tenth cc. of different dilutions of filtrate was injected intracutaneously at different sites in order to determine the allergic response to various quantities of Brucella protein. The reactions were read at 24-hour intervals, and the extent of erythema and edema was measured. The reactions were usually at their height after 48 to 72 hours, from which time on they receded, persisting in some instances for as long as 10 to 14 days.

[20]Calmette, A., Négre, L., and Boquet, A.: Manual of microbiological and serological technic (Masson et Cie., Paris, 1926). Cited by I. F. Huddleson in "Brucellosis in man and animals," p. 239.

EXPERIMENTS

Sensitization of Rabbits and Guinea Pigs.—A suspension of a 72-hour culture of *Br. abortus* was prepared in saline and adjusted to compare with tube 3 of the McFarland nephelometer. Four groups of rabbits were used. Ten animals each were injected intravenously with 1 cc. of the bacterial suspension; ten others were injected with 1 cc. of the suspension intraperitoneally; five animals received intravenous injections of 1-cc. doses of the suspension which had been acted upon by phenol in a concentration of 0.5 per cent for 24 hours, and five rabbits received intravenous injections of 1-cc. doses of the suspension which had been heated in a water bath at 60 C. for one

TABLE II—Skin Reactions in Guinea Pigs in Response to Intracutaneous Injection of Sonic Filtrate

Guinea Pig Number		Allergic Skin Reactions After Injection of Brucella Abortus									
		48 Days					85 Days				
		Dilution of Protein in Filtrate					Dilution of Protein in Filtrate				
		1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:500	1:1,000	1:2,000	1:4,000	1:8,000
503	Injected with live Brucella intraperitoneally	+3	+3	+3	+2	+2	+4	+4	+3	+3	+2
505		+2	—	—	—	—	*				
506		+3	+3	+2	+2	+1	+2	+2	+2	+1	+1
507		+3	+3	+2	+2	+1	+2	+2	+1	+1	—
508		+4	+4	+3	+3	+2	*				
509		+3	+2	+2	+1	—	+3	+2	+2	+2	+1
510		+2	+2	+2	+1	—	*				
511		+2	+2	+1	—	—	*				
512		+3	+3	+1	—	—	+3	+2	+2	+2	+1
513		+2	+2	+1	—	—	*				
514	Noninjected Controls	—	—	—	—	—	—	—	—	—	—
515		—	—	—	—	—	—	—	—	—	—
516		—	—	—	—	—	—	—	—	—	—
517		—	—	—	—	—	—	—	—	—	—
518		—	—	—	—	—	—	—	—	—	—
519							—	—	—	—	—
520							—	—	—	—	—
521							+2	+1			

*Died before second test. – No reaction. +1, Erythema and edema up to 5 mm. in diameter. +2, Erythema and edema 5 to 10 mm. in diameter. +3, Erythema and edema 10 to 15 mm. in diameter. +4, Erythema and edema 15 to 20 mm. in diameter.

TABLE III—Agglutinative, Precipitative and Opsonocytophagic Tests 2½ Months After injection of Br. Abortus

Rabbit Serum No.		Agglutinative Tests								Precipitative Tests								Opsonocytophagic Tests			
		Dilutions of Serums								Dilutions of Sonic Filtrate According to Its Protein Content								Degree of Phagocytosis			
		1:25	1:50	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:500	1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000	1:64,000	1:128,000	Marked	Moderate	Slight	Negative
600		+4	+4	+4	+4	+4	+4	+3	—	+	+	+	+	+	+	+	—	—	7	10	6	2
601		+4	+4	+4	+4	+3	—	—	—	+	+	+	+	+	+	—	—	—	8	10	7	—
602		+4	+4	+4	+4	+4	+4	+1	—	+	+	+	+	+	+	+	—	—	13	7	5	—
603		+4	+4	+4	+4	+4	+4	+2	—	+	+	+	+	+	+	+	—	—	10	9	6	—
604		+4	+4	+4	+4	—	—	—	—	+	+	+	+	—	—	—	—	—	11	9	5	—
606		+4	+4	+4	+4	+4	+4	+4	—	+	+	+	+	+	+	+	+	—	14	7	4	—
607		+4	+4	+4	+4	+4	+4	+2	—	+	+	+	+	+	+	+	+	—	12	7	6	—
608		+4	+4	+4	+4	+4	+3	—	—	+	+	+	+	+	+	+	—	—	15	6	4	—
609		+4	+4	+4	+4	+4	+4	+1	—	+	+	+	+	+	+	+	+	—	13	10	2	—
610		+4	+4	+4	+4	+4	+4	+3	—	+	+	+	+	+	+	+	+	—	1	6	14	4
612		+4	+4	+4	+4	+4	+4	+2	—	+	+	+	+	+	+	+	—	—	9	6	8	2
613		+4	+4	+4	+4	+4	+1	—	—	+	+	+	+	+	+	—	—	—	7	9	9	—
614		+4	+4	+4	+4	+4	+4	+1	—	+	+	+	+	+	+	+	—	—	2	16	7	—
615		+4	+4	+4	+4	+4	+4	+2	—	+	+	+	+	+	+	+	—	—	9	11	5	—
616		+4	+4	+4	+4	+3	—	—	—	+	+	+	+	+	+	—	—	—	5	11	9	—
617		+4	+4	+4	+4	+4	+2	—	—	+	+	+	+	+	+	—	—	—	5	9	10	1
618		+4	+4	+4	+4	+3	—	—	—	+	+	+	+	+	+	+	—	—	13	5	7	—
620		+4	+4	+4	+2	—	—	—	—	+	+	+	+	—	—	—	—	—	10	12	3	—
621		+4	+4	+4	—	—	—	—	—	+	+	—	—	—	—	—	—	—	5	9	7	4
624		+4	+4	+4	—	—	—	—	—	+	+	—	—	—	—	—	—	—	4	8	8	5
625		+4	+4	+4	+4	+3	+1	—	—	+	+	+	+	—	—	—	—	—				
626		+4	+4	+4	+4	+4	+4	+1	—	+	+	+	+	—	—	—	—	—				
627		+4	—	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	10	3	5	7
628		+4	+4	+4	+4	+2	—	—	—	+	+	+	+	—	—	—	—	—				
629		+4	+4	+4	+3	—	—	—	—	+	+	+	+	—	—	—	—	—	5	6	9	5
638	Nonimmune Controls	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	14	11
639		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	12	13
640		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13	12
641		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	10	10
642		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	20

Agglutinative tests: – Negative; +1 to +4, different degrees of agglutination.
Precipitative tests: – Negative; +, precipitate at bottom of tube.
Degree of phagocytosis: Negative, when no phagocytosis occurs. Slight, when from one to 20 bacteria are found in the cell. Moderate, when from 21 to 40 bacteria are found in the cell. Marked, when the number of bacteria in the cell is above 40.

hour. A part of the phenolized and a part of the heated suspension were cultured on tryptose agar at the time of injection, and it was found that the organisms in the phenol-treated suspension were dead, while in the heated suspension not all of the bacteria were killed, probably due to the high density and the presence of bacterial clumps in the suspension. Boak and Carpenter[21] found that *Br. abortus* was killed at 140 to 142 F. (60 to 61.1 C.) in 15 minutes.

At the same time guinea pigs were injected intraperitoneally with single 1-cc. doses of a 72-hour *Br. abortus* culture which had been suspended in saline to compare with tube 10 of the McFarland nephelometer. Preliminary agglutinative tests were conducted on the rabbits and guinea pigs and all of the blood serum proved negative in a dilution of 1:5.

The rabbits and guinea pigs were skin-tested 48 and 85 days after injection. The skin reactions recorded in tables I and II are those observed 48 hours after the intracutaneous injections of sonic filtrate. These results indicate that the rabbits injected with live organisms intravenously (Nos. 600 to 609) showed the best skin reactions. Not one animal in that group of rabbits reacted negatively to the allergic skin test. In the next group (rabbits 610 to 618), which had been injected with live organisms intraperitoneally, two rabbits were negative (Nos. 612 and 617) in the first test, and one rabbit (No. 612), which was still negative in the second test, never developed an allergic skin sensitivity. Rabbits 620 to 624, which had been injected with a phenol-killed culture, were not actually sensitized to the skin test and their reactions were comparable with those of the control animals. In the group of rabbits (Nos. 625 to 629) which had been injected with a heated but not entirely killed suspension of organisms, a considerable degree of sensitivity was observed in three of the animals. An intraperitoneal injection of live organisms into guinea pigs resulted in a definite skin sensitivity to sonic filtrate.

[21]Boak, R. A., and Carpenter, C. M.: The thermal death point of *Bacterium abortus* in milk. J. Inf. Dis., xliii (1931), p. 327.

In a few of the guinea pigs central necrosis was observed 72 to 96 hours after injection of the lower dilutions of filtrate. An injection of 0.1 cc. of a 1:8,000 to 1:16,000 protein dilution (the highest dilutions used in the two tests) appeared sufficient to produce a skin reaction in sensitized rabbits and guinea pigs. In subsequent tests a 1:32,000 and sometimes a 1:64,000 dilution yielded good results.

The serologic tests conducted on the rabbits demonstrated that the agglutinative titers were highest one month after the injection of *Br. abortus*, receding gradually from that time. The groups of rabbits which received, respectively, intravenous and intraperitoneal injections of live culture had the highest agglutinative titers; the group injected intravenously with heated culture ranked second, and the group injected intravenously with phenol-killed culture ranked third. The precipitative titers corresponded in general to the agglutinative titers of the serums. The opsonocytophagic tests demonstrated clearly that an injection of Brucella organisms increased the degree of phagocytosis considerably as compared with the control animals, whose blood showed only slight phagocytic ability. In table III are recorded the results of the agglutinative, precipitative and opsonocytophagic tests carried out 2½ months after the injections of Brucella organisms, and prior to the second intracutaneous tests.

By comparing different lots of sonic filtrate on the basis of their protein content it was found that 0.1-cc. doses of each lot with the same protein concentration produced similar skin reactions when injected intracutaneously into a sensitized animal.

ABILITY OF SONIC FILTRATE TO SENSITIZE ANIMALS TO SUBSEQUENT SKIN TESTS AND TO STIMULATE ANTIBODY PRODUCTION

Control rabbits used in previous skin tests developed agglutinins specific for Brucella antigen following intracutaneous injections of sonic filtrate. The agglutinative titer and the length of time for which it persisted was directly proportionate to the quantity of protein injected. Multiple injections totaling 0.4 mg. of protein resulted in agglutinative titers up to 1:400 two weeks after the intracutaneous in-

jections, and about seven months were necessary for the agglutinins to disappear. On the other hand, injections totaling 0.15 mg. of protein were followed by an initial agglutinative titer of 1:50 which disappeared in about three months, so that the blood serum was negative in a 1:5 dilution. In addition, two of the control animals gave a slight skin reaction when retested after one month. However, these animals had received intracutaneous injections of rather high concentrations of sonic filtrate, *i.e.*, 0.2 to 0.4 mg. of protein.

In order to investigate this problem further, an experiment was carried out on rabbits and guinea pigs to determine the ability of sonic filtrate to sensitize animals to subsequent intracutaneous tests. Twenty-seven rabbits and 18 guinea pigs, divided into three equal groups, were used for this purpose. Nine rabbits and six guinea pigs in the first group each were injected intracutaneously with 0.1 cc. of a 1:2,000 protein dilution of filtrate (0.05 mg. of protein); the second group received 0.1 cc. of a 1:4,000 dilution of protein (0.025 mg.) and the third group 0.1 cc. of a 1:8,000 dilution of protein (0.0125 mg.). These animals were retested at regular intervals with the corresponding doses of protein concentration.

Agglutinative tests also were carried out to ascertain the curve of agglutinin concentration in the blood serums. Four rabbits and three guinea pigs in each group were retested four, seven and ten weeks after the initial intracutaneous injections, and of the 21 animals only one guinea pig showed a slight edematous swelling 48 hours after the skin injections in the first and second retests. In the third retest, ten weeks after the original intracutaneous injection, this guinea pig was negative. However, it is noteworthy that this animal belonged to the group which had been injected and retested each time with a 1:2,000 dilution of protein, which was the highest concentration used. The remaining five rabbits and three guinea pigs in each group were retested two months after the initial intracutaneous injection of sonic filtrate, and they were all negative. Finally, a retest of the rabbits and guinea pigs with the corresponding doses of sonic filtrate 5½ months after the initial injection yielded completely negative results.

Table IV presents the agglutinative titers of three groups of rabbits injected with different concentrations of sonic filtrate. Aside from the individual variation in the capacity to produce agglutinins, it is apparent that the animals in group 3, which had received the highest dilution of protein, developed a comparatively low agglutinative titer which disappeared sooner than in the other two groups. The concentration of agglutinins in group 2 diminished faster than in group 1. In general, it took about eight to ten weeks for agglutinins to disappear from the blood serum of rabbits which had been injected with 0.1 cc. of a 1:2,000 protein dilution of sonic filtrate. Intracutaneous injections of sonic filtrate also stimulated the production of opsonins as determined by opsonocytophagic tests.

DISCUSSION

The studies on sonic filtrate of *Br. abortus* showed that it stimulated an allergic response when injected intracutaneously into sensitized rabbits and guinea pigs. The dilutions of filtrate used in the skin tests were based upon the protein content, and the fact that the same concentrations in different lots of filtrate produced very similar skin reactions would indicate that the protein constitutes the active allergic substance. In some instances appreciable reactions were obtained by the injection of 0.1 cc. of as high as 1:64,000 protein dilution in the filtrate. Rabbits injected with live *Br. abortus* intravenously developed the best skin sensitivity. Those injected with live culture intraperitoneally and the groups of rabbits injected with heated but not killed culture intravenously showed variable results. The rabbits injected with phenol-killed culture intravenously were not sensitized to the skin test.

In addition to its allergic activity, sonic filtrate also bound precipitins, as shown in the precipitative tests. Serologic tests carried out on the infected animals demonstrated that an injection of *Br. abortus* into rabbits stimulated the production of agglutinins, precipitins and opsonins. The

TABLE IV—Agglutinative Reactions in Rabbits in Response to Injection of Different Concentrates of Sonic Filtrate

Rabbit Number	Agglutinative Reactions After Injections of Sonic Filtrate	One Week						Two Weeks						Three Weeks						Four Weeks						Six Weeks						Seven Weeks					
		1:5	1:10	1:25	1:50	1:100	1:200	1:5	1:10	1:25	1:50	1:100	1:200	1:5	1:10	1:25	1:50	1:100	1:200	1:5	1:10	1:25	1:50	1:100	1:200	1:5	1:10	1:25	1:50	1:100	1:200	1:5	1:10	1:25	1:50	1:100	1:200
724	Injected with 0.1 cc. of 1:2,000 dilution of protein	4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
726		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
727		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
728		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
729		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
735	Injected with 0.1 cc. of 1:4,000 dilution of protein	4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
736		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
737		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
739		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
740		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
749	Inj. with 0.1 cc. of 1:8,000 dilution of protein	4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
750		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
751		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		
752		4	4	4	3	2	1	4	4	4	2	1		4	4	4	1			4	4	3	2			4	4	3	3			4	4	3	2		

+1 to +4, Different degree of agglutination. —Negative.

phenolized culture produced low agglutinative and precipitative titers, but no skin sensitivity.

Since intracutaneous injections of sonic filtrate had been found to stimulate agglutinin production in nonsensitized animals, an experiment was carried out to ascertain how long the agglutinins would persist in the blood serum and whether an intracutaneous injection of the filtrate could sensitize an animal to a subsequent skin test.

Twenty-seven rabbits and 18 guinea pigs were divided into three equal groups. One group of each species was injected intracutaneously with 0.1 cc. of a 1:2,000 protein dilution of sonic filtrate (0.05 mg. of protein), the second group received 0.1 cc. of a 1:4,000 dilution of protein (0.025 mg.) and the third group 0.1 cc. of a 1:8,000 dilution of protein (0.0125 mg.). Half the number of animals was retested with the corresponding doses of protein concentration 4, 7, 10 and 24 weeks after the initial intracutaneous injections. The remaining rabbits and guinea pigs were retested at 2 and at 5½ months after the initial injections. Of all the animals used, only one guinea pig showed a slight edematous swelling at the site of injection when retested after four and again after seven weeks. In the subsequent two retests no reactions were noticed. This guinea pig, however, belonged to the group which had been injected each time with the highest concentration of protein, namely, a 1:2,000 dilution.

The agglutinative titers were highest one week after the intracutaneous injection of sonic filtrate and were directly proportional to the concentration of filtrate used. It took eight to ten weeks for the agglutinins to disappear from the blood serum of rabbits which had received the largest doses, *i.e.*, 1:2,000 protein dilution. The smaller the quantity of filtrate injected, the lower was the agglutinative titer and the sooner it disappeared.

SUMMARY

Sonic filtrate of *Brucella abortus* evoked skin reactions in sensitized rabbits and guinea pigs. The degree of reaction seemed

to depend upon the protein concentration in the filtrate.

An intravenous injection of live *Br. abortus* into rabbits produced the best skin sensitivity to sonic filtrate. Animals injected with live culture intraperitoneally and those injected with heated but not killed organisms intravenously were less uniformly sensitized, while a phenol-killed culture injected intravenously did not produce an allergic skin sensitivity.

The groups of rabbits which received, respectively, intravenous and intraperitoneal injections of live culture had the highest agglutinative titers, the group injected intravenously with heated culture ranked second, and the group injected with phenol-killed culture ranked third. Precipitins and opsonins were also present in the blood serums of the injected animals.

Intracutaneous injections of single doses of sonic filtrate into control rabbits and guinea pigs did not sensitize these animals to subsequent skin tests after 4, 7, 10 and 24 weeks. However, the intracutaneous inoculations stimulated the production of agglutinins. The initial agglutinative titer and the length of time it persisted depended upon the concentration of filtrate used.

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Feeding as a Contributory Factor in the Development of Chronic Mastitis*

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IT IS UNIVERSALLY agreed by research workers that the majority of mastitis cases are associated with streptococcic infections. *Streptococcus agalactiae* is the organism most generally encountered. Less frequently mastitis may be associated with other streptococci, staphylococci, coliform organisms and miscellaneous types.

The common portal of entry is generally thought to be the teat canal. However, considerable difficulty has been encountered with artificial transmission experiments. Attempts to transmit the disease under natural conditions, such as by milking with contaminated hands or by exposing the ends of the teats to infective material, usually have given negative results. If the ends of the teats are injured during the period of exposure, the results are more apt to be positive. Yet, cases of mastitis develop in cows where no history of visible teat injury has been observed. On the other hand, animals with severe teat injury may not necessarily develop chronic mastitis. Repeated exposures are often necessary to establish the infection in an apparently normal cow, even though relatively large experimental doses of the organisms are introduced into the udder.

With this difficulty encountered in explaining the transmission of mastitis, investigations have been made to discover some factor or factors predisposing to the development of mastitis.

REVIEW OF LITERATURE

Munch-Petersen[1] made an extensive review of mastitis literature up to 1935 in which he cited the work of Frost, Heinke, Proschaldt, Richter, Raebiger, Wiedeman, Gatgen and Wesche published in European journals, as well as work by Udall[3] and Johnson in this country, in which it was found that feeding, particularly alterations in the composition of the feed, predisposed to the development of mastitis.

Wiedmann[2] gave the results of a number of experiments in which he was able to change the composition of the milk, where the milk sugar was low and the salt content high, to a milk having normal composition and properties, by changing the character of the rations. A deficiency of carbohydrates in the feed was found to bring about a definite change in the chemical composition of the milk.

Udall and Johnson[3] found that intensive feeding with protein concentrate was detrimental. They stated: "When an owner states that a certain cow made a high record but was 'burned out' by high feeding and never produced well again, one is usually justified in the opinion that she has mastitis." Frost[4] said: "The streptococcus is the principal factor in mastitis in cows. There are other contributing or predisposing factors, however, such as cold, traumatism, a high protein feed and all conditions that lower the resistance of the udder. The latter conditions, however, do not of themselves produce mastitis. We once tried to induce mastitis with a high protein ration. The animal lived on cottonseed meal. There was no effect on the udder. After about one year the animal was destroyed and neither macroscopically nor microscopically could any injury be found as a result of the feed. We then made the same experiment with gluten meal, with the same results. When this was tried in slightly infected herds, many of the animals quickly came down with mastitis."

Hotis and Woodward[5] used eight cows to study the effect of heavy cottonseed-meal feeding. These cows had shown udder troubles in

*Scientific paper No. 272 of the West Virginia Agricultural Experiment Station; presented before the Section on Research at the seventy-eighth annual meeting of the American Veterinary Medical Association, Indianapolis, Ind., August 11-15, 1941.

[1]Munch-Petersen, E.: Bovine mastitis. Imperial Bureau of Animal Health, Weybridge, Surrey, England (Rev. Ser. No. 1, 1935).

[2]Wiedmann, F.: Mastitis and carbohydrate deficiency (title trans.). Zeit. f. Untersuch. Lebensmittel, lxiii (1932), p. 113. Cited by George W. Cavanaugh in Proc. 29th Ann. Conv. Int. Assn. Milk Dealers (1936).

[3]Udall, D. H., and Johnson, S. D.: Diagnosis and control of mastitis. Cornell Vet., xxi (1931), p. 190.

[4]Frost, J. N.: Prevention and treatment of mastitis. Vet. Med., xxix (1934), p. 205.

[5]Hotis, R. P., and Woodward, T. E.: Heavy cottonseed meal feeding in relation to udder troubles in dairy cows. USDA Tech. Bul. 473 (1935).

the past. All were fed alfalfa hay. Five to 10 lb. of a low-protein grain mixture was fed to four cows. The other four received 10 lb. of cottonseed meal each day. The experiment lasted 22 weeks. The conclusion drawn in this work was that liberal feeding on a high-protein ration composed of cottonseed meal and alfalfa hay had little if any influence on the abnormality of the milk. Neither did such a ration aggravate udder conditions as determined by physical examination of the udders and by laboratory examination of the milk, nor did the high-protein ration force animals, more or less subject to chronic attacks of mastitis, into clinical cases.

PLAN OF EXPERIMENTS

Corn is one of the principal constituents of the concentrate ration fed many dairy cows. Some dairymen are of the opinion that heavy corn feeding may be associated with udder disturbances. Since there is lack of agreement in the literature available on the relationship of feeding to the development of chronic mastitis, it seemed desirable to make a further study of the effect of heavy corn feeding and the rate of feeding as possible predisposing factors. With this in view the following experiments were conducted.

EXPERIMENT I. EFFECT OF HEAVY CORN FEEDING

Animals Used.—When the experiment was started, 20 milking animals were available, including 17 registered Holsteins and 3 registered Guernseys. On the basis of the number of lactations, production and previous evidence of mastitis, the animals were divided into comparable experimental and control groups. During the investigation it became necessary to remove two of the animals in the control group and four in the experimental group. They were replaced by first-lactation Holstein heifers. All cows were free of tuberculosis and Bang's disease throughout the period of study in both experiments.

Rations.—The control group was fed a grain ration composed of ground oats 400 lb., wheat bran 300, yellow corn meal 150, corn-gluten feed (24% protein) 100, cottonseed meal (41% protein) 50, salt 15, and steamed bone meal 10 lb. This ration contains approximately 12.4 per cent digestible crude protein and 71.5 per cent total digestible nutrients.

The experimental group was fed a grain ration composed of yellow corn meal 600 lb., corn-gluten feed 350, salt 15, and steamed bone meal 10 lb. This ration contains approximately 12.5 per cent digestible crude protein and 77.4 per cent total digestible nutrients.

The experiment was continued for a period of two years. All animals were fed legume hay and silage and had access to pasture in season. The grain rations were fed at the rate of 1 lb. of grain to each 3.5 lb. of milk.

Method of Management.—The groups were managed as one herd. No attempt was made to separate them in stalls or milk them separately. However, on the basis of the various diagnostic tests to be described later, the cows were classified as negative, suspicious and positive. They were milked in the same order twice daily, starting with the negative animals. The cows were bred to calve as near as possible at intervals of twelve months preceded by a dry period of eight weeks.

Diagnostic Tests.—In order to detect the presence of mastitis and to study the progress of its development the following diagnostic tests were conducted at two-week intervals: (1) physical examination, (2) strip cup, (3) brom-thymol blue, (4) chlorine determination (colorimetric), (5) Hotis test, (6) leucocyte count, (7) microscopic examination and (8) blood-agar plates. In conjunction with the above tests during the latter half of the experiment, chemical determinations were made at monthly intervals on one-half pint samples of fore milk from each quarter for chlorine (titration method), lactose and casein number.

The physical examination was made immediately after the udder was milked out, according to the technic described by Udall and Johnson.[5] The strip cup (with 100 squares to the inch) was used twice daily as a routine practice. The chlorine determination was made by a colorimetric method described by Hayden.[6]

The Hotis test was conducted according to the technic described by Hotis and Miller.[7] For making the leucocyte count and microscopic examination the same slide was used. Preparation consisted of placing 0.01 cc. of incubated milk (12 to 14 hours) on a slide covering approximately one square centimeter of surface. The slide was stained according to the Newmann-Lampert[8] method.

Blood-agar plates were made by adding 10 per cent defibrinated horse blood to a bactoveal agar. Frequently both cow blood and horse blood were used in bactoveal agar to compare the difference in hemolytic activity of the organism encountered. The medium contained 50 Gm. of bactoveal, 10 Gm. of bactopeptone,

[6]Hayden, C. E.: Field tests for chlorine in milk for the detection of mastitis. Cornell Vet., xxii (1932), p. 277.

[7]Hotis, R. P., and Miller, W. T.: A simple method for detecting mastitis streptococci in milk. USDA Circ. Bul. 400 (1936).

[8]Standard methods for the examination of dairy products. Am. Pub. Health Assn. (7th ed., 1939), p. 53.

TABLE I—Effect of Heavy Corn Feeding on the Development of Chronic Mastitis

Herd No.	No. Lactations	Time on Project				Production		
		Starting Date	Reaction	Ending Date	Reaction	Milk	Fat	Days
Control Group—Normal Herd Ration								
G-168	11	8–10–37	Clinical	8–11–38	Subclinical	7,708	325	287
H-305	6	8–10–37	Clinical	3– 7–39	Clinical	12,650	341	287
H-347	5	8–10–37	Negative	3–23–39	Subclinical	12,206	376	305
H-358	4	8–10–37	Clinical	1–10–39	Clinical	8,382	296	286
H-360	5	8–10–37	Negative	7–11–39	Subclinical	15,168	423	305
G-384	6	8–10–37	Negative	6–10–39	Subclinical	6,081	293	305
H-389	4	8–10–37	Negative	3–23–39	Subclinical	7,948	315	305
H-408	2	8–10–37	Negative	3– 9–39	Clinical	11,166	339	305
H-416	3	8–10–37	Negative	7–11–39	Clinical	9,842	309	305
H-427	2	9– 9–37	Negative	3–23–39	Clinical	9,519	316	305
H-453	1	7–26–38	Negative	5–30–39	Clinical	7,306	263	305
H-455	1	12–27–38	Negative	7–11–39	Subclinical	8,480	281	286
Experimental Group—Corn and Corn-Gluten-Feed Ration								
H-176	9	8–10–37	Clinical	5–31–38	Clinical	15,178	490	305
G-341	9	8–10–37	Clinical	12– 5–38	Subclinical	7,510	355	286
H-355	4	8–10–37	Clinical	3–23–39	Subclinical	14,872	422	305
H-361	4	8–10–37	Clinical	5–30–39	Clinical	14,023	446	305
H-362	5	8–10–37	Negative	2–22–39	Subclinical	9,421	367	287
H-373	5	8–10–37	Negative	7–11–39	Acute	11,943	376	305
H-390	3	8–10–37	Negative	8–23–38	Negative	10,245	335	305
H-413	3	8–10–37	Negative	5–16–39	Clinical	9,662	291	289
H-424	2	8–10–37	Negative	7–11–39	Clinical	12,388	440	305
H-438	1	12–13–37	Negative	10–18–38	Negative	8,597	281	305
H-442	1	7–12–38	Negative	5–30–39	Subclinical	9,605	306	305
H-443	1	9– 7–38	Negative	5–30–39	Subclinical	9,665	334	295
H-445	1	8–11–38	Negative	7–11–39	Subclinical	10,695	338	305
H-464	1	10– 4–38	Negative	7–11–39	Subclinical	7,302	259	305

G = Guernsey. H = Holstein.

15 Gm. of agar, 10 Gm. of sodium chloride and 1,000 cc. of distilled water. The sterilized medium was adjusted to a pH of 6.8. A 1:20 dilution of milk in physiologic salt solution was added to poured blood-agar plates. Plates were incubated for 24 hours at 37 C., then placed under refrigeration for an additional 24 hours in accordance with Brown's[9] recommendation to bring about the development of double-zone beta hemolytic streptococci colonies.

Chemical analyses were conducted in the manner described in previous work.[10]

Cows showing positive on the strip cup regularly or intermittently, along with fibrosis of the udder and supported by the bacteriologic findings and chemical changes in the composition of the milk, were diagnosed as clinical cases. The presence of hemolytic streptococci accompanied by an increased leucocyte count and changes in the chemical composition of the milk were diagnosed as subclinical cases.

Results and Discussion

The results obtained with the two groups of cows are summarized and presented in table I. It may be observed that three animals in the control group and four in the experimental group showed clinical symptoms of chronic mastitis at the beginning of the experiment. It also will be noted that these cows had been in production four or more lactations.

At the conclusion of the experiment the twelve animals in the control group were diagnosed as six clinical and six subclinical

[9]Brown, J. H.: Double-zone beta hemolytic streptococci. J. Bact., xxvii (1934), p. 84.

[10]VanLandingham, A. H., Weakley, C. E. Jr., Moore, E. N., and Henderson, H. O.: Mastitis. I. The relationship of the development of mastitis to changes in the chlorine, lactose and casein number of milk. J. Dairy Sci., xxiv (1941), p. 383.

cases. Four animals in the experimental group were diagnosed as clinical and eight subclinical cases, with two remaining negative. One of the cows (373) diagnosed as subclinical had an acute attack during August 1938. This cow remained in the herd until July 1939. Before this attack and after recovery she did not develop clinical symptoms. During the acute attack the affected quarter was milked out and hot applications applied at approximately 20-minute intervals for about eight hours, at the end of which the milk appeared normal and the swelling had diminished. Samples taken at two-hour intervals during the day showed marked improvement from time to time, and microscopic examinations failed to reveal the presence of bacteria. However, two colonies of hemolytic streptococci were present during the succeeding test. For several months before the conclusion of the experiment, this quarter was diagnosed as negative, whereas two other quarters were diagnosed as subclinical.

There appeared to be no significant difference in the incidence or severity of mastitis between the groups of cows receiving the heavy corn ration and a normal grain ration as fed in this experiment.

EXPERIMENT II. EFFECT OF RATE OF GRAIN FEEDING

Animals Used.—When this experiment was started in the fall of 1939, eleven purebred Holstein heifers at the beginning of their first lactation were available. They were divided into a control and an experimental group. Six were placed in the control group and five in the experimental group. Since that time five similar first-lactation heifers have been added, two to the control group and three to the experimental group, making a total of eight in each group. Six of the heifers in the control group and five in the experimental group are near the end of their second lactation, whereas the five animals which were added to the project are near the end of their first lactation.

Ration.—Both groups were fed the same ration as the control group in the first experiment, with the exception that 50 lb. of soybean oil meal replaced 100 lb. of corn-gluten feed

TABLE II—Effect of the Rate of Grain Feeding on the Development of Chronic Mastitis

Herd No.	No. Lactations	Time on Project				Production*		
		Starting Date	Reaction	Ending Date	Reaction	Milk	Fat	Days
Control Group—1 lb. of Grain to 3.5 lb. of Milk								
H-469	2	9–12–39	Negative	In herd	Clinical	3,872	236	305
H-474	2	8–10–39	Negative	In herd	Negative	7,569	299	305
H-481	2	8–10–39	Negative	In herd	Subclinical	7,403	266	305
H-485	2	9–12–39	Negative	Sold 4–14–41	Clinical	8,122	297	305
H-486	2	9–12–39	Negative	In herd	Clinical	7,419	286	305
H-508	2	5– 8–40	Negative	In herd	Negative	7,540	279	276
H-511	1	1– 6–41	Negative	In herd	Clinical	Incomplete		
H-517	1	3–17–41	Negative	In herd	Negative	Incomplete		
Experimental Group—Limited Grain Feeding—8 to 10 lb. Daily								
H-475	2	8–24–39	Negative	Died 11–28–40	Negative	9,764	349	297
H-482	2	8–10–39	Negative	In herd	Clinical	6,697	242	305
H-490	2	8–10–39	Negative	Sold 4–14–41	Subclinical	8,513	270	305
H-492	2	8–24–39	Negative	Sold 6–18–41	Clinical	7,350	247	305
H-510	1	1– 6–41	Negative	In herd	Negative†	Incomplete		
H-512	1	2–16–40	Negative	In herd	Clinical	Incomplete		
H-518	1	1– 6–41	Negative	Sold 7–10–41	Clinical	Incomplete		
H-483	2	8–10–39	Negative	Sold 4–14–41	Clinical	9,072	323	305

*First lactation record. †Fourth quarter blind. H = Holstein.

(24%), the difference between the control and the experimental group being the rate and method of grain feeding. The control group was fed the grain ration at the rate of 1 lb. of grain for each 3.5 lb. of milk. The experimental group was fed a limited amount of grain during lactation. This group received from 8 to 10 lb. of grain per day, depending upon production, throughout the experiment. The aim was to have the animals come into lactation in good condition; then, by following a limited grain-feeding schedule, they would not be forced to their maximum capacity for milk production during the peak of production, as is the case when heavy-producing animals are fed 1 lb. of grain for each 3.5 lb. of milk, regardless of the level of production. By this system of feeding, both groups received the same amount of grain when the production level was between 28 and 35 lb. of milk per day. Above 35 lb. the limited-grain-fed group received proportionately less grain than the control group. Below 28 lb. near the end of lactation, the limited-grain-fed group received more grain in proportion to the milk produced than did the control group. This procedure was varied for a brief period when the animals were dry and for three weeks after gestation, in accordance with the conventional method of feeding dairy cows.

Method of Management.—The animals were handled as one herd in the manner described for the first experiment.

Diagnostic Tests.—Methods of diagnosis were practically the same as those described for the first experiment.

RESULTS AND DISCUSSION

The results obtained with the two groups of cows are summarized in table II. Attention is called again to the fact that all animals were negative first-calf heifers when placed on the experiment. There were four animals in the control group and five in the experimental group which showed clinical symptoms of chronic mastitis. One animal in the control group and one in the experimental group were subclinical cases. Three control animals and two experimental animals were negative. One of the animals (510) freshened with a weak quarter which, after a brief period, failed to function.

There appears to be no significant difference between the two groups of cows, depending upon the rate of grain feeding, in this experiment. It may be observed that the animals used in this experiment were started as first-lactation heifers and produced only moderate amounts of milk. Nevertheless, several of them developed clinical symptoms of mastitis during the second lactation.

SUMMARY AND CONCLUSION

A study was made of 26 animals to determine the effect of heavy corn feeding as a contributory factor in the development of mastitis, and 16 animals to determine the effect of rate of feeding the grain ration.

No appreciable difference was noted in the incidence or severity of mastitis between the experimental and control lots. Physical, biologic and chemical tests demonstrated wide variation in the severity of mastitis among individuals, but not between the two groups.

In conclusion, it can be said that no appreciable increase in the incidence or severity of mastitis was observed as a result of the kind of ration or the rate of grain feeding under the conditions of these experiments.

The Relation of Leucemia and Bovine Lymphocytoma*

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THE NOMENCLATURE OF the malignancies involving the blood and blood-forming organs is indeed chaotic. Feldman[1] commented on the confusing terms used to identify these pathologic entities. If the term sarcoma, for example, is used to designate a malignant connective tissue tumor, there is little justification for the term lymphosarcoma for neoplasms of lymphoid cells. Pseudoleucemia is an illustration of another term which implies too much and describes too little, yet Bengston[2] cites its use in routine meat-inspection pathologic usage. One eminent pathologist expressed it thus, "It is difficult in this group of conditions to determine where leucemia leaves off and sarcomas begin."

Too rarely is there an opportunity for veterinarians to study in detail the cases involving these conditions over a period of time. They are seen on an occasional call, at autopsy or on the killing floor. The purpose of this paper, therefore, is to attempt to clarify this situation by correlating some observations on lymphocytoma in cattle.

Associated with the unrestrained rate of reproduction of the lymphoid or myeloid tissues are the changes in the circulating blood which may or may not attain distinct leucemic proportions. Too seldom have there been careful hematologic studies correlated with the pathology which is present. The excellent work of Feldman[1] deals essentially with the tissue cytologic aspects, with little regarding the hematology. Knuth[3] mentions, however, that one of the most striking characteristics of lymphocytomatosis is the marked increase of lymphocytes in the blood associated with the tumors and infiltrations and that in severe cases of lymphocytomatosis, the percentage of lymphoid cells increases to as high as 96 per cent of all of the leucocytes. A preliminary report on the frequent association of a leucemia with a high proportion of immature lymphocytes in the blood which was correlated with neoplastic changes of the lymph tissues was made by Frank and Thompson.[4]

A simple classification of leucemia from its hematologic aspect may be accepted as lymphogenous, myelogenous and monocytic.

Various writers have commented on the greater frequency of lymphogenous leucemia as contrasted with the rarity of the myelogenous form; moreover, diagnosis of the occasional case of myelogenous leucemia in animals has been made essentially on postmortem pathologic evidence rather than by hematologic examinations. Forkner[5] prefers to use the term leucemia for identifying conditions already described by well-defined terminology rather than to emphasize the neoplastic feature as has Feldman.[1] From the studies at this laboratory it seems logical to conclude that the leucemic feature and the neoplastic tissue aspect are but two manifestations of the same fundamental process.

BLOOD STUDIES

Table I represents the hematologic examinations of ten cases in which the diagnosis of lymphocytomatosis was confirmed on autopsy. It appears that the total white cell county may vary from near normal (6,300) to a marked leucemia (142,500). The majority of cases, in which a series of blood counts have been obtained, tend to show

*Contribution No. 90 from the Department of Pathology, Division of Veterinary Medicine, Kansas State College; presented before the Section on Research at the seventy-eighth annual meeting of the American Veterinary Medical Association, Indianapolis, Ind., August 11-15, 1941.

[1]Feldman, W. H.: Neoplasms of domestic animals (W. B. Saunders Co., Philadelphia, 1932).

[2]Bengston, J. S.: Primary reticulum sarcoma of the lymph nodes of a cow. Am. J. Path., xiv (1938), pp. 365-376.

[3]Knuth, P.: Die Lymphozytomatose des Rindes. Arch. f. Wiss. u. Prakt. Tier., xliii (1916-17), p. 129.

[4]Frank, E. R., and Thompson, W. W.: Lymphoblastoma in the bovine. No. Am. Vet., xxi (April 1940), pp. 217-221.

[5]Forkner, C. E.: Leukemia and allied disorders (The Macmillan Co., New York, 1938).

TABLE I—Blood Counts on Ten Cases with Postmortem Diagnosis of Lymphocytomatosis

ANIMAL	DATE OF EXAMINATION	EOS.	BAS.	JUV.	STAB	SEG.	LYMPHOCYTES			MONO.	WHITE CELLS	RED CELLS	HEMOGLOBIN*
							L	A	S				
Ayrshire Bull, 10 years old	6–17–40	12	0	0	3	34	6	21	24	0	8,850	4,850,000	11.0
	6–28–40	6	1	0	27	45	4	11	3	3	14,750	4,750,000	8.5
	7–13–40	13	1	0	3	46	7	24	4	2	7,600	2,750,000	Not read
Jersey Cow	8–13–40	1	0	0	7	38	12	23	14	5	9,700	5,500,000	10.0
	8–15–40	0	1	0	10	42	1	13	33	0	11,800	5,450,000	9.0
	8–20–40	0	0	0	17	21	6	44	7	5	6,300	4,875,000	9.0
Hereford Cow, 4-5 years old	8–17–40	0	0	0	7	5	3	83	1	1	142,500	3,250,000	7.5—
	8–20–40	0	0	0	1	4	12	83	0	0	100,000	2,500,000	7.5—
Shorthorn Cow, 6 years old	9–16–40	0	0	0	13	25	34	19	1	8	9,400	5,087,500	11.0
	9–17–40	2	0	0	10	25	13	32	2	16	10,700	6,300,000	11.0
	9–18–40	1	0	0	5	25	12	55	2	0	12,800	6,350,000	12.0
	9–20–40	0	0	0	9	30	18	35	5	3	8,000	6,500,000	12.0
Jersey Cow, 6 years old	9–23–40	0	0	0	5	25	5	57	2	6	20,000	5,175,000	9.5
	9–25–40	0	0	0	10	26	0	55	7	2	22,600	5,300,000	10.5
Hereford Steer, 1 year old	2– 5–41	0	0	0	0	26	56	13	4	1	24,000	11,950,000	14.0
	2–10–41	0	0	0	1	11	80	5	2	1	11,600	9,050,000	11.0
	2–18–41	0	0	0	0	14	71	14	1	2	16,000	10,500,000	12.0
	3– 7–41	0	0	0	0	5	47	47	0	1	10,800	10,000,000	11.0
	3–17–41	0	0	0	0	11	46	41	2	0	11,500	9,500,000	11.0
	3–27–41	0	0	0	0	12	38	50	0	0	8,800	8,600,000	10.0
	4–15–41	0	0	0	0	17	43	40	0	0	8,000	8,300,000	9.5
Jersey Cow, 6 years old	2–12–41	0	0	1	6	10	12	59	11	1	12,900	4,662,000	8.0
	2–13–41	0	0	2	11	12	12	58	5	0	23,900	5,265,000	8.5
	2–14–41	0	0	0	3	27	16	46	6	2	18,300	5,000,000	9.0
Hereford Steer, 2 years old	2–26–41	0	0	14	15	16	10	37	7	1	8,600	6,500,000	8.5
Holstein Cow, 5 years old	4– 8–41	3	0	3	13	15	20	20	26	0	9,200	2,150,000	7.5—
	4–11–41	1	0	0	9	26	8	48	1	7	18,600	1,750,000	7.5—
Grade Jersey Cow, 5 years old	4– 9–41	1	0	2	9	27	2	8	51	0	Total Counts Not Made		
	4–17–41	1	0	0	17	44	8	23	5	2	13,200	4,600,000	7.5—

L = Large. A = Atypical. S = Small. *Expressed in grams per 100 cc.

that as the disease progresses and death approaches, the total white cell count decreases gradually from a distinct leucemia to a normal or subnormal level. In attempts to diagnose lymphocytomatosis by the blood count, the chief reliance should be placed on the percentage of lymphocytes in the differential count and particularly on the percentage of the lymphocytes that are classified as atypical. An analysis of the differential counts of the 29 examinations made on the ten cases included in the above table shows an average of 65.24 per cent of lymphocytes; of these, 31.4 per cent are large lymphocytes, 56.2 per cent are atypical and 12.4 per cent are small lymphocytes. Differential counts approaching or exceeding these figures for lymphocytes should leave little doubt as to the accuracy of a diagnosis of lymphocytomatosis. In cases where the picture is less definite a series of several counts and a careful study of the clinical history should be made before a diagnosis is rendered.

The red cell counts in table I average slightly over 5,900,000 per cubic millimeter of blood, which is slightly less than 1,700,000 below the normal average. This average low red cell count with many bloods showing a much reduced hemoglobin content* indicates that a mild to a marked anemia is associated with these cases.

Polycromasia and stippling of the red cells are frequently found in the stained blood films. Poikilocytosis and the presence of large numbers of macrocytes and microcytes are constant. No platelet counts have been made, although they appear to be much more numerous on the blood films from cases of lymphocytomatosis than they are on similar preparations from normal animals or animals suffering from other diseases. These factors should be considered when attempting to arrive at a diagnosis in cases that do not show an outstanding differential white cell count. These

*The Haden-Hausser hemoglobinometer was used for these determinations.

observations on the hematology of lymphocytomatosis agree with the general conclusions of Knuth.[3]

FREQUENCY OF OCCURRENCE

Udall[6] in his extensive experience has encountered only two cases of leucemia and doubts that it is a common disease of domestic animals. Many animals with indefinite symptoms and with extensive internal lesions show no external visible enlargements of the lymph nodes; hence, such cases might easily be overlooked if a hematologic examination is not made.

Observations at this laboratory during the past three years on the occurrence and pathology of this condition indicate that, for this area at least, it has been the most common malignant neoplasm of cattle. This is in agreement with Feldman,[1] who comments that if all animal species are considered, the lymphoblastomas probably occur with greater frequency than any of the other neoplasms which affect lower animals and that lymphocytoma is the most common one in cattle. Through the routine hematologic examination for diagnosis of the puzzling cases in cattle in the clinic during the past three years, a total of 111 animals have been found which showed an increase in the blood of atypical lymphocytes.* Of that number, 38 came to autopsy, 36 of which had gross lesions of lymphocytoma. There is a high correlation, therefore, between the blood picture and the presence of lymphocytoma. The remaining cases were returned to the farm, whence some were sold on the market and some died; in other cases no further examination was made or they are still under observation. Furthermore, specimens have come to this laboratory from time to time on which a tentative diagnosis of carcinoma was made but which show the prevailing cells to be lymphoid rather than epithelial.

MYELOGENOUS LEUCEMIA

Forkner[5] suggested that the oxidase reaction is needed for the differentiation of lymphogenous and myelogenous leucemia. The trials with the oxidase staining methods in this work were not satisfactory, although the blood and tissues from some of the cases of lymphocytoma seemed to be oxidase negative.

The characteristic finding at autopsy in myelogenous leucemia is the enormous enlargement of the spleen in contrast to the size of that organ in lymphocytoma. The finding of erythroblasts and megacaryocytes in the spleen is further evidence of the myelogenous nature of the process.

[6]Udall, D. H.: The practice of veterinary medicine (3rd ed., 1939), pp. 235-237.

*Lymphocytes that show altered staining or morphologic characteristics are classified as atypical. The most common changes in staining characteristics are cells that take too little stain, appearing pale blue with only a slight differentiation between nucleus and cytoplasm, and cells that take more than a normal amount of stain in the cytoplasm. Morphologically the most predominate change is to a large-type cell with a bizarre-shaped nucleus and many large vacuoles in the cytoplasm. Less common are lymphocytes containing two or more nuclei and others that show several nucleoli.

Fig. 1. Bovine spleen. Myelogenous leucemia.

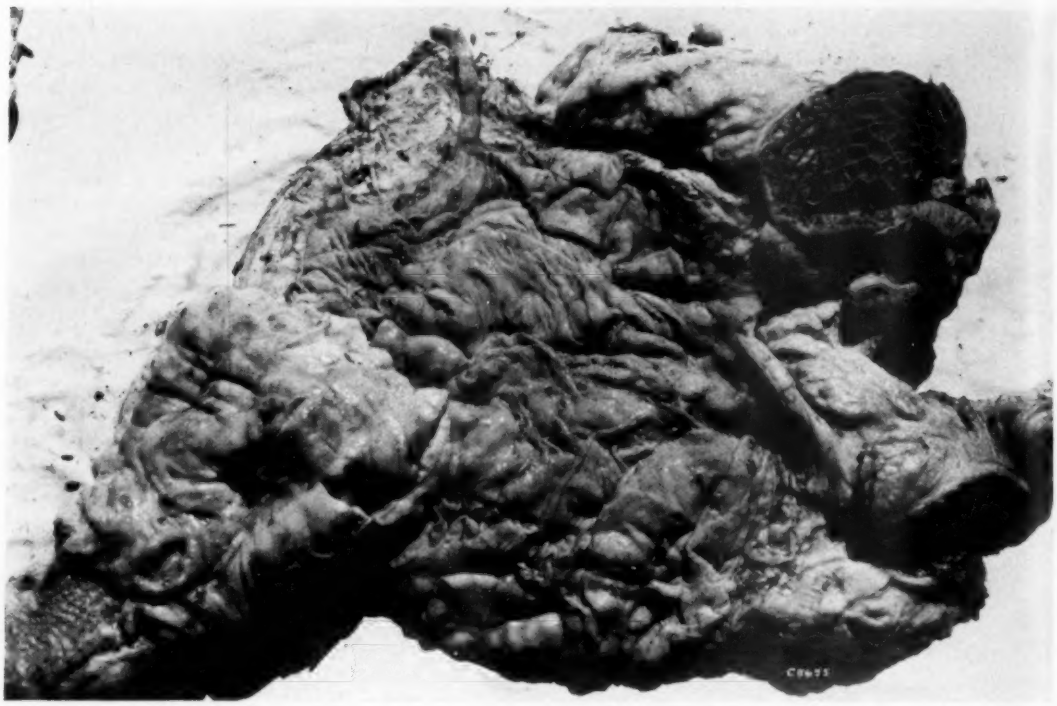

Fig. 2. Bovine abomasum, omasum and reticulum.

There is little enlargement of the lymph nodes in this form of leucemia. Few authenticated cases of myelogenous leucemia have been reported in animals. Langham and Hallman[7] examined a porcine spleen which was involved in myeloid leucemia. Feldman[1] noted that the spleen was involved in four hogs in his series of tissues and that in one of the spleens large, multinucleated giant cells were present.

Three spleens obtained from animals apparently affected with myelogenous leucemia were sent to this laboratory through the coöperation of the U. S. Bureau of Animal Industry at Kansas City, Kan. One hog spleen was enormously enlarged. It was so soft that it could hardly be handled without rupture of the capsule. The lymph nodes were but slightly enlarged. There were clumps of nucleated red cells and megacaryocytes in the spleen. Another specimen (fig. 1) from a 750-lb. cow weighed 22 lb. and measured 34½x10½x4 in. It was soft and somewhat lighter in color than normal; the cut surface showed many small, gray nodules which were fairly definitely circumscribed and up to 4 mm. in diameter. Nucleated red cells were fairly numerous, although megacaryocytes seemed to have been absent. There is seldom any enlargement of the spleen in cases of lymphocytoma; one exception was found in which it was 2 to 2½ times its normal size although the differential blood count showed 91 to 98 per cent of lymphocytes.

[7]Langham, R., and Hallman, E. T.: Chronic myeloid leukemia of a hog. J.A.V.M.A., xciv (1939), p. 653.

PATHOLOGY OF LYMPHOCYTOMA

One of the most significant features of lymphocytomatosis is the varied distribution, extent and neoplastic character of the lesions. The essential pathology is the obliteration of the normal architecture of lymphoid tissue and its replacement by a diffuse hyperplasia of immature lymphocytes. There is the invasion of tissues, ordinarily containing few lymphocytes, by infiltrating masses of the neoplastic cells and the destruction of normal tissue cells. All of this is characteristic of a neoplastic

process. At times the lymphoid hyperplasia is confined to enlargement of existing lymph nodes. A lymph node which may normally be no larger than 3 or 4 cm. in length may enlarge to 10 to 12 cm. in diameter. At times the diffuse enlargement is confined to the nodes adjacent to the infiltrating lymphocytoma in some organ. The nodes are enlarged at times sufficiently to exert apparently enough pressure on the anterior gluteal and great sciatic nerves to cause paralysis. Many times there are new growths and rounded nodular masses whose essential origin from definite lymph nodes can not be determined. An example was the finding of an irregularly oval growth which measured approximately 10.6 x 11.8 x 19.6 in. and which weighed 53 lb. It surrounded and lay posterior to the heart. Sharply circumscribed nodules are seen occasionally in the lungs and kidneys, suggesting an embolic origin if not in actuality the presence of cells capable of multiplication and neoplastic activity in the circulating blood.

Little connective tissue framework is found in the microscopic structure of the lymphocytoma. Such tissue is usually quite soft, of a pale yellow, grayish white, to a pale pink color. Hemorrhage takes place at times into the tumors and necrosis of the interior of the larger masses is common. The appearance of the cells varies from normal appearing lymphocytes to a decidedly more embryologic aspect. Such cells have a large vesiculate granular nucleus which stains lightly, and there is little discernible cytoplasm. Mitotic figures are seen at times.

The wall of the abomasum is one of the more common sites of predilection for lymphocytoma. Feldman[1, 8] has described this characteristic lesion in detail. It has been found invading the muscular layer, in contrast to his observations. The wall has been found to have attained a thickness of 2 in. Ulceration of the mucous membrane is common.

Of two extreme examples of involvement of the abomasum, the mucosa of one (fig. 2) showed numerous folds, ulcers and many nodules. When opened, it measured 14 x 22 in. and weighed 17.9 lb. There was a roll-shaped mass about 3 or 4 in. in diameter at the attachment of the omentum in the other specimen, which measured 20 x 34 in.

Fig. 3. Lymphoid tumor mass surrounding omasum.

when opened. There were extensive infiltrations in the walls of the adjacent omasum and reticulum at their junction and the three compartments weighed 32.1 lb. An omasum (figs. 3 and 4) with its surrounding tumor tissue weighed 32.2 lb. and measured 15 x 19 in., and in places it had attained a thickness of 6 in. The reason for the persistent bloating in that animal was apparent.

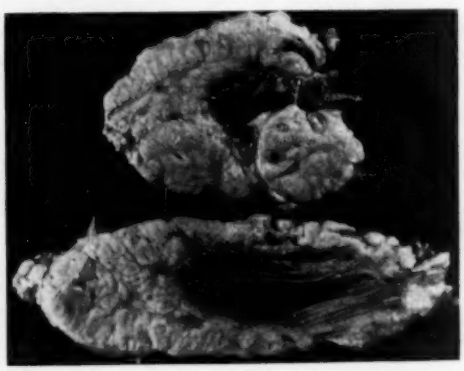

Fig. 4. Cut section of omasum shown in figure 3.

The small intestine seems to be involved more rarely than the abomasum, although nodular lymphoid masses 2.5 cm. in diameter have been found in the wall. The mesenteric lymph nodes are occasionally in-

[8]Feldman, W. H.: Lymphosarcoma of the bovine abomasum. J.A.V.M.A., lxxiii (1928), pp. 206-215.

volved in the hyperplastic process. The liver may show circumscribed tumor nodules or a diffuse infiltration of the lymphoid cells with consequent injury to the liver parenchyma. A case illustrative of that condition was a mule with the dorsal border of the liver extending back nearly to the

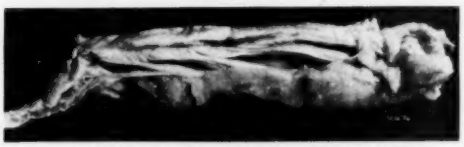

Fig. 5. Bovine cauda equina surrounded by lymphocytoma.

kidneys; the weight of the liver was 35 lb. The presence of the lymphocytomatous masses in the orbit with the consequent exophthalmos and blindness has been mentioned by a number of writers.[1, 2, 4]

Animals affected clinically with lymphocytoma frequently exhibit symptoms of nerve involvement. This may be manifested by paralysis of the limbs or by any of the obscure symptoms produced by lesions affecting the central nervous system. In our studies the most common symptom of nervous dysfunction was a bilateral posterior paralysis induced by a tumorous mass either in the pelvic cavity or dorsal region of the abdominal cavity which exerted pressure on a nerve trunk. In several cases these masses were found within the vertebral canal, causing pressure on the cord (fig. 5). One very interesting case was that of a 10-year-old bull in which the obscure symptoms had led to a number of different clinical diagnoses until a blood count suggested lymphocytoma. The postmortem findings were negative until the cranium was opened, where a large lymphocytoma 3 x 4 x 1 in. was found in the posterior-dorsal region of the cavity (fig. 6). The nerve tissue seldom is invaded by the adjacent neoplastic process.

The heart, too, is often involved. Pink to pale gray nodules and diffusely infiltrated areas are common. Marked enlargement is sometimes encountered. The cardiac muscle fibers lose their staining properties and disappear when such invasion occurs. In the lungs the process usually assumes the appearance of circumscribed metastatic tumor nodules.

The genitourinary system may likewise be involved in the malignant process. The kidney lesions may be either diffuse infiltrations or circumscribed nodules. The kidneys and bladder wall of a hog contained a large number of circumscribed nodules which were bright red with hemorrhage. The major calyx of a bovine kidney was filled with lymphoid tissue and one ureter was surrounded by a mass of similar structure. The uterus of a cow which had a white cell count of 16,350 measured 50 in. over the greater curvature of one horn and 23 in. over the other. The wall of the body was about 1 in. thick while that of the horn consisted of about 0.5 in. of lymphoid tissue.

SUMMARY

The term lymphocytoma seems to be the most appropriate designation for that neoplastic process of animals primarily involving lymphoid cells and tissues. There seems to be little justification for a more complicated nomenclature to supply a more descriptive terminology for variations in fundamentally the same malignant condition. Observations indicate, moreover, that lymphocytomatosis is probably the most common neoplasm of cattle and that if adequate means of recognition are utilized, it may be found to constitute a problem of economic significance.

The neoplastic process is more or less regularly characterized by a varying degree

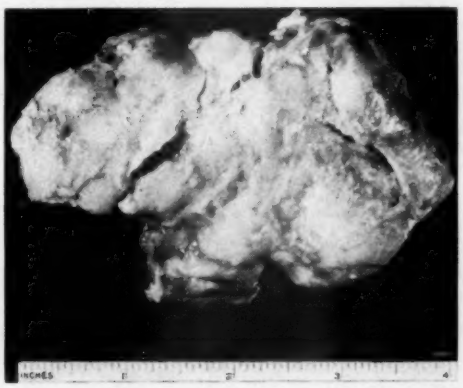

Fig. 6. Lymphocytoma from bovine cranial cavity.

of leucemia while the differential white cell count quite constantly shows a large proportion of atypical lymphocytes in the circulating blood. The experience at this laboratory indicates that a total white cell count and a differential leucocytic count in cases of obscure bovine ailments usually will aid in diagnosing the condition. The pathology of the occasional cases of myelogenous leucemia is described.

ACKNOWLEDGMENTS

Assistance in dealing with the clinical aspects of this study was rendered by Drs. E. R. Frank and S. J. Roberts of the Department of Surgery and Medicine, Kansas State College. The coöperation of the inspectors of the Bureau of Animal Industry at Kansas City, Kan., in submitting characteristic specimens was likewise of especial aid.

Listerella Infection in Cattle*

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IN RECENT YEARS extensive researches have been conducted on a variety of diseases occurring in various species of animals and man apparently caused by small, gram-positive bacteria. These organisms have been known under various names, including *Bacterium monocytogenes* and *Listerella hepatolytica;* at present they are listed in Bergey's Manual under the genus Listerella. Pirie suggested that Listerella be changed to Listeria, but the latter designation is obviously not an improvement, and to avoid further confusion we will continue to use the term Listerella until a more acceptable one is adopted in the official classification of bacteria.

Listerella infection in cattle varies considerably from that in other species. Generalized gross lesions which are observed in human beings, chickens and rodents usually are absent in infected cattle, sheep and swine. The number of cattle in a herd which are affected in an outbreak is comparatively small. The percentage of recoveries is much higher and losses are lower compared with those in other species. It is evident from our experiences with the transmission of this disease under experimental conditions that cattle are comparatively resistant to Listerella infection. The data secured from both field and experimental studies indicate that sheep are very susceptible to listerellosis while swine and cattle are far more resistant.

*From the Veterinary Research Institute, Iowa State College.

REVIEW OF LITERATURE

The literature contains a considerable number of references to Listerella infection in cattle. Some of the earlier reports included descriptions of symptoms and pathologic changes found in the central nervous system identical with those found in listerellosis, but classified the disease as an encephalitis. In 1928, Mathews[1] reported a case occurring in feeder calves which undoubtedly was due to Listerella infection, although he failed to mention the recovery of the etiologic agent. In 1934, Jones and Little[2] recovered a gram-positive bacterium from cattle which were affected with an encephalitis. Fincher[3] reported an outbreak of meningo-encephalitis from which gram-positive organisms were isolated. The following year, 1936, Olafson[4] made extensive investigations on this disease of cattle from which small, gram-positive organisms were cultured. In 1938, Graham and his associates[5] reported several outbreaks of listerellosis in cattle in various herds in Illinois. The latter[6] also reported observations on other cases in 1940.

REPORT OF A CASE

The first Iowa field case of Listerella infection in cattle was encountered by us

[1]J.A.V.M.A., lxxv (1928), p. 513.
[2]Arch. Path., xvi (1934), p. 580.
[3]Cornell Vet., xxv (1935), p. 61.
[4]The practice of veterinary medicine (D. H. Udall, Ithaca, N. Y., 2nd ed., 1936), p. 113.
[5]Sci., lxxxviii (1938), p. 171.
[6]Cornell Vet., xxx (1940), p. 97.

on March 7, 1938. Two steers in a herd of feeders developed severe central nervous disturbances following a period of depression and loss of appetite. One steer died and postmortem examination showed no gross lesions in the heart, liver, lungs, spleen or lymph nodes. The eyes were clear and there was no evidence of a digestive disturbance. The brain was brought to the laboratory for examination. It showed only a slight congestion with a few petechiations in the cerebellum and medulla. There was also a slight softening apparent in the medulla and spinal cord. Cultures made direct from the brain were negative. Frozen sections prepared for histopathologic study revealed perivascular and focal areas of infiltration consisting of accumulations of monocytes and polynuclear leucocytes in the cerebellum.

Transmission experiments were made on two rabbits. A 10 per cent brain suspension in sterile saline solution was made for intracranial inoculation. Rabbit A was inoculated with 0.25 cc. of a Berkefeld N filtrate of this suspension. No unfavorable reaction was observed. Rabbit B was given an intracranial inoculation of 0.25 cc. of the unfiltered brain emulsion. This animal developed a severe central nervous disturbance and died two days following inoculation. Cultures made from the liver and spleen were negative, but cultures made direct from the brain on liver-infusion agar were later identified as a variety of Listerella.

The other steer was found in the feedlot on March 14, lying on its left side and unable to arise. It exhibited symptoms of a severe central nervous disturbance accompanied by pronounced salivation. When it was turned over on its right side, it immediately returned to its original position on its left side. It died during the night of March 15. No significant lesions were found in the internal organs. Cultures made from the brain on liver-infusion agar were positive. Histologic examination of the brain tissue showed advanced focal and perivascular infiltration. Intracranial inoculation of brain emulsion into rabbits produced symptoms of a severe central nervous disturbance terminating in death about 40 hours after inoculation. Pure cultures of the same organism were secured on liver-infusion agar. Histologic examination of the rabbit brain tissue revealed similar pathologic changes.

Many sporadic outbreaks of this disease in cattle have since been reported throughout the state, a large number of which have been confirmed by laboratory diagnosis. These cases have been reported during all seasons of the year. However, our records show the greatest incidence in spring and early summer. The disease has appeared sporadically in widely separated areas and apparently affects only a comparatively small number of animals in a herd. The severity of the disease as manifested by clinical symptoms varies greatly. Some affected animals showing mild clinical symptoms apparently make a complete recovery in a short time. Recovery in some of the more severe cases is slower and may never be complete. The animals which show severe reactions and are unable to remain on their feet seldom recover.

EXPERIMENTAL PROCEDURE

Cultures of the organisms obtained from the field cases were maintained by frequent transfer on suitable culture mediums and sealed in tubes to prevent undue desiccation. The disease was readily transmitted to rabbits and guinea pigs by both intracranial and intravenous injection of suitable bacterial suspensions made from the cultures grown on liver-infusion agar. The early attempts to transmit this disease to cattle by intracranial inoculation were unsuccessful. There appeared to be a slight reaction for two or three days following the inoculation. During this period there was a transient rise in temperature, slight depression and decreased appetite followed by apparent recovery. However, the experimental animals did not show normal growth and development following recovery.

The results of our preliminary studies on the transmission of this disease to cattle indicated that they were considerably more resistant to this infection than were rab-

bits or guinea pigs. It was considered probable that the method of inoculation might be a factor in transmitting this disease to cattle and that repeated injections might be more effective. Seven calves weighing between 300 and 350 lb. were selected for this experiment. Two calves were injected intracranially, two intravenously and two were given culture suspensions orally. One calf was injected subcutaneously. The cultures used (Nos. 984 and 1054) were secured from field cases and were maintained on liver-infusion agar. Saline suspensions of these Listerella cultures were made (density No. 2 tube McFarland nephelometer) for experimental transmission.

Intracranial Inoculation. — Calf 1 was inoculated intracranially with 1 cc. of the suspension of culture 984 and subsequently showed a slight general reaction. This was followed by a slight increase in temperature for three days, reaching a high of 104.2 F. Slight depression and decreased appetite also were observed. The calf apparently recovered but did not show the normal growth and development usually observed.

Calf 2 was inoculated intracranially with 1 cc. of the suspension of culture 1054. The response to the inoculation was similar to that of calf 1, with a maximum temperature reaction of 105.2 F. on the fourth day. It apparently made a complete recovery and was observed for a period of three months, after which it was destroyed and examined for pathologic lesions. Significant gross lesions were not present. The characteristic histopathologic changes were not found. Cultures made from the brain, liver, spleen, kidneys and lymph nodes were negative. A 10 per cent brain suspension was inoculated into a rabbit. There was no apparent reaction and the rabbit was released after observation of one month.

Because of failure to produce a positive reaction upon the first inoculations in the other calves, repeated doses of bacterial suspensions were administered periodically in an attempt to transmit the disease. The procedure is summarized in table I.

Intravenous Inoculation.—Calf 3 was given a series of intravenous inoculations of culture 984. The first inoculation, administered on March 17, was followed by a mild general reaction. There was a slight elevation of temperature for three days, the highest reaching 104.2 F. on the second day. The appetite was diminished for several days and there was an increase in the consumption of water. On the fourth day the calf was considerably more active and

TABLE I—Transmission Experiments in Calves with Repeated Doses of Bacterial Suspensions

Date	Intravenous Inoculation		Oral Administration		Subcutaneous Inoculation
	Calf 3 Culture 984	Calf 4 Culture 1054	Calf 5 Culture 984	Calf 6 Culture 1054	Calf 7 Culture 1054
3–17–39	20 cc.	20 cc.	250 cc.	250 cc.	20 cc.
4– 5–39	20 cc.	20 cc.	200 cc.	200 cc.	20 cc.
4–13–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
4–19–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
4–21–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
4–24–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
4–26–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
4–28–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
5– 1–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
5– 3–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
5– 5–39	10 cc.	10 cc.	200 cc.	200 cc.	10 cc.
5–10–39	20 cc.	20 cc.	200 cc.	200 cc.	10 cc.
5–12–39	20 cc.	20 cc.	200 cc.	200 cc.	20 cc.
5–15–39	20 cc.	20 cc.	200 cc.	200 cc.	20 cc.
5–17–39	20 cc.	20 cc.	200 cc.	200 cc.	20 cc.
5–19–39	20 cc.	20 cc.	200 cc.	200 cc.	20 cc.

appeared normal seven days following the first inoculation. Because of the failure to establish a typical case of listerellosis in these experimental calves, it was decided to administer a series of intravenous inoculations in an attempt to reproduce a reaction similar to that observed under field conditions. This series of subsequent inoculations was started on April 5 and continued until May 19. No further reaction was observed during the course of this procedure. Observations were continued until June 23 and no abnormal symptoms were noted. The animal did not show normal growth and development and the hair coat appeared rough and dry.

Calf 4 was given intravenous inoculations of culture 1054. This animal showed a more severe reaction than calf 3 during the six days following the initial inoculation. The highest temperature recorded was 105.6 F. It gradually subsided to normal on the ninth day. Large amounts of water were consumed during this period, but little food was eaten for the first five days. The appetite gradually increased and the calf appeared quite normal again on the eighth day following the initial inoculation. Beginning April 5, a subsequent series of inoculations were given like those administered to calf 3. No further reaction was observed, but the calf showed the same retarded growth and development as observed in calf 3.

This animal was kept under observation until June 23, when it was destroyed and examined for pathologic lesions. No gross lesions were observed in the brain, liver, spleen, kidneys or lymph nodes and cultures made from these organs were negative. A 10 per cent brain emulsion made with sterile physiologic saline solution was inoculated intracranially into a rabbit. The results were likewise negative. The rabbit was observed until July 13 and released. Histologic studies of organs taken from the calf showed no significant changes.

Oral Administration.—Calf 5 was given an initial dose of 250 cc. of bacterial suspension made from culture 984. This material was administered by way of the mouth through a stomach tube. No general reaction was observed and no thermic reaction was recorded. The calf was then given a series of doses orally from April 5 to May 19. No reaction was observed, but the general appearance of the animal was not quite as good as a normal calf of that age.

Calf 6 was given a similar dose of bacterial suspension of culture 1054. On the third day following, a temperature reaction was observed reaching 105 F. There was a noticeable general reaction and no food was consumed from the fourth to the sixth day. The temperature gradually subsided and the calf appeared normal by the eighth day. The same series of doses were administered from April 5 to May 19 as were given to calf 5. No further reactions were observed and the calf appeared to be in as good condition as calf 5.

This animal was under observation until June 23, at which time it was destroyed and examined for pathologic changes. No gross lesions were observed in the brain, liver, spleen, kidneys or lymph nodes and cultures made from these organs were negative. A rabbit inoculated intracranially with a brain emulsion from this calf showed no reaction. The histologic studies made on the tissues of this calf showed no significant pathologic changes.

Subcutaneous Inoculation.—Calf 7 was given a subcutaneous inoculation of 20 cc. of bacterial suspension of culture 1054 on March 17. No general reaction was observed nor any appreciable thermic reaction recorded. This was followed by a series of inoculations from April 5 to May 19. No reactions were noted during this period.

Observations were continued until the animal was destroyed on June 23. Gross pathologic changes were not observed in the brain, liver, spleen or lymph nodes and cultures made from these organs were negative. Intracranial inoculation of a 10 per cent brain emulsion into a rabbit was also negative. The rabbit was kept under observation until July 13 and released. Histologic studies of the organs taken from

the calf showed no significant pathologic changes.

PATHOGENICITY OF BACTERIAL CULTURES

The analysis of the experimental data secured from this series of animal inoculations with cultures 984 and 1054 seemed to indicate that the calves inoculated with the latter showed a more severe reaction. In neither instance was a typical or fatal infection observed. Even though the preliminary attempts to transmit the infection indicated that cattle are more resistant than either sheep or swine to listerellosis, it seemed advisable to check the virulence of these organisms on rabbits which were used to isolate the organisms from the field cases. The original culture 978 was isolated March 7, 1938, and culture 1054 was secured from a field case on January 17, 1939. At the time they were isolated on artificial mediums, 0.2 cc. of bacterial suspension inoculated intracranially in a rabbit would prove fatal in 24 hours or less. This was also true of culture 979 isolated from a field case on March 16, 1938.

After the strains had been cultivated on mediums over a considerable period, they showed a marked tendency to decreased virulence. Rabbits inoculated with 0.2 cc. of bacterial suspension of culture 978, held under laboratory conditions for some time, failed to show a fatal reaction. Culture 1054 likewise produced only a mild reaction on rabbits while culture 979, also inoculated intracerebrally, produced a fatal infection in four days. The latter culture was used to make serial passages through 14 rabbits, following which the virulence of this organism increased considerably, producing a fatal infection in 20 hours after the fourteenth rabbit passage.

TRANSMISSION BY INTRACRANIAL INOCULATION FOLLOWING RABBIT PASSAGE

Three steer calves weighing approximately 350 lb. each were inoculated intracranially with 1 cc. of bacterial suspension of culture 979. The virulence of this organism had been restored by serial passage through 14 rabbits.

Calf 8, inoculated intracranially on March 28, showed a general reaction the following day with a temperature of 104.2 F. It consumed large quantities of water but ate no food. The calf was found dead on the morning of March 30. Postmortem examination revealed an excessive quantity of yellowish pericardial fluid, petechiae on the epicardium, firm but light colored heart muscle, liver light mahogany color and enlarged, brain and meningeal tissue edematous and turbid. Cultures from the brain were positive whereas those from the liver, spleen and kidney were negative.

The microscopic changes found in this calf were similar to those seen in calves 9 and 10, but they were less severe. In this connection, it should be noted that the infection in this case terminated fatally in about 36 hours.

Calf 9 also was inoculated intracranially on March 28 with 1 cc. of a suspension of culture 979. A slight reaction was observed the following day and a temperature of 104 F. was recorded. On the morning of March 30, the calf was found in a moribund condition and was destroyed. The gross pathologic changes found on postmortem examination were similar to those found in calf 8. The reaction in the brain appeared more severe and excessive quantities of fluid were found in the ventricles. Cultures from the brain were positive. Cultures from the liver, spleen, kidneys and heart blood were negative.

The microscopic changes observed in this calf consisted of a severe infiltrative meningitis, perivascular cuffing with monocytes and some polymorphonuclear leucocytes. The perivascular infiltrations were more severe and occurred more frequently in the vessels near the periphery and in the subependymal regions. The nerve cells were characterized by early degenerative changes. Foci of monocytes and increased cellular infiltrations also were observed.

Calf 10 likewise was inoculated intracranially with a suspension of culture 979 on March 28. It showed considerable reaction the following day, the temperature rising to 104.8 F. The central nervous symptoms developed progressively and the

temperature gradually became lower. The temperature reactions were as follows:

Date	*Temperature*
3-29-40	104.8
3-30-40	104.0
3-31-40	105.0
4- 1-40	103.8
4- 2-40	103.2
4- 3-40	103.2
4- 4-40	100.4

On March 3, the calf was unable to rise and appeared to be in a comatose condition, with respirations somewhat irregular. It was moribund on the morning of March 4 and was destroyed. Significant gross lesions other than considerable congestion and edema in the brain tissue were not observed in the organs of this animal. Cultures from the brain were positive, but cultures from the liver, spleen, kidneys and lymph nodes were negative.

The histologic changes found in the brain were more advanced than the alterations encountered in the two previous calves. The severity and the distribution of the microscopic lesions of this case resembled those found in some field cases of bovine listerellosis. Many blood vessels in the deeper parts of the brain presented advanced perivascular infiltrations. Extensive areas of focal and diffuse infiltrations consisting chiefly of monocytes with many polymorphonuclear cells were found (figs. 2 and 3).

Intravenous Inoculation in Cattle

Two grade dairy cows were selected for intravenous inoculation of culture 979. Cow 11 was in production and cow 12 in advanced pregnancy. They were inoculated on March 28 with 10 cc. of a suspension made from culture 979, the pathogenicity of which had been increased by serial passage through rabbits.

Cow 11 showed a severe general reaction the day following inoculation. She refused feed and water and appeared extremely nervous and irritable. She consumed some water on the third day and ate some grain and hay on the fourth day. The initial temperature reaction was severe and progressively decreased to normal level. The temperature reaction was as follows:

Date	*Temperature*
3-29-40	106.0
3-30-40	104.2
3-31-40	104.6
4- 1-40	101.0
4- 2-40	102.6
4- 3-40	101.8
4- 4-40	100.0
4- 5-40	99.0
4- 6-40	98.6

The milk production dropped off sharply the day following the intravenous inoculation. It remained low for four days and gradually increased for twelve days, at which time it reached a constant level somewhat lower than before the intravenous inoculation. Attempts to isolate the Listerella organism from the milk on culture mediums were unsuccessful during the entire course of the reaction. The animal recovered and appeared in good physical condition. However, the milk production was considerably decreased.

Cow 12 was given an intravenous inoculation on March 28 of 10 cc. of a suspension of culture 979. This cow did not show the same initial reaction as observed in the case of cow 11. The animal became greatly depressed, spending the major part of the time lying quietly in her stall. Food and water were not taken for two days. On the third day she drank some water. She started eating again on the fourth day and gradually regained her normal appetite. The initial temperature rise was not quite

CAPTIONS TO ILLUSTRATIONS ON OPPOSITE PAGE

1) Listerella monocytogenes (var. bovis). Gentian-violet stain (x 990).

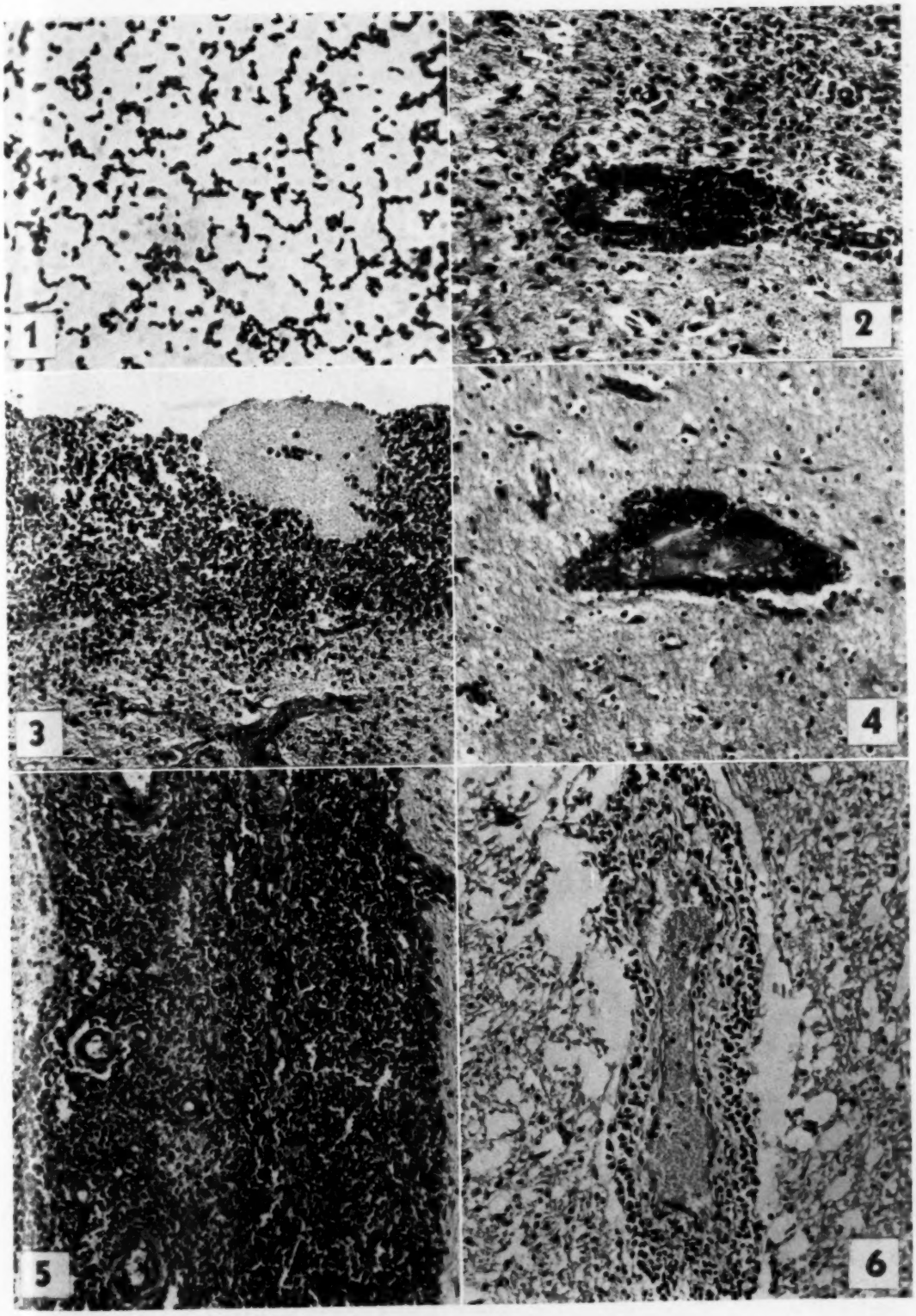
2) Brain of calf, showing perivascular cuffing and diffuse infiltrations. Experimental infection (x 150).

3) Cerebral ventricle of calf, showing destruction of ependymal lining, infiltration by monocytes and polymorphonuclear cells. Experimental infection (x 150).

4) Perivascular reaction in brain stem of pig. Experimental infection (x 150).

5) Experimental meningitis in a pig (x 150).

6) Perivascular infiltration in brain of sheep. Experimental infection (x 150).


1
2
3
4
5
6

as great as that of cow 11. The temperature reaction was as follows:

Date	*Temperature*
3-29-40	105.4
3-30-40	103.2
3-31-40	101.2
4- 1-40	101.4
4- 2-40	101.0
4- 3-40	101.2
4- 4-40	100.6

This animal made a complete recovery and quickly regained her normal weight and condition. On April 7, she gave birth to a strong, healthy calf. She had no difficulty calving and the subsequent milk production was apparently normal.

Inoculation of Bovine Listerella in Swine

Intracranial Inoculation. — Three pigs weighing approximately 100 lb. were inoculated intracranially with 1 cc. of a suspension of culture 979 on March 28. Pigs 1 and 2 died on the morning of March 29 and pig 3 died on the morning of March 30. The livers were slightly congested and somewhat friable. The hearts showed mild degenerative changes. There was some congestion in the meningeal and submeningeal tissues of the brain. There was no gross hemorrhage or necrosis evident in the brain, but excessive fluid was found in the ventricles. Cultures made from the various organs, including heart blood, liver, spleen, kidneys and lymph nodes, were negative in all three animals, but positive cultures were obtained from the brain tissue in each case.

The histopathologic findings in the three pigs were similar. A severe meningitis appeared, as in other experimental cases which died in 36 hours or less following intracranial inoculations. However, perivascular cuffing was found in many vessels in deeper locations. Degenerative changes of blood walls and hemorrhages also were noted (figs. 4 and 5).

Intravenous Inoculation. — Two pigs weighing about 125 lb. were inoculated intravenously with 5 cc. of a suspension of culture 979. Pig 5 showed a more severe initial reaction than pig 4. Some evidence of depression accompanied the temperature reaction. The appetite decreased for several days. The temperature reactions were as follows:

Date	*Pig 4*	*Pig 5*
3-29-40	104.6	106.0
3-30-40	104.0	105.0
3-31-40	104.2	104.4
4- 1-40	101.0	102.2
4- 2-40	102.0	102.4
4- 3-40	101.4	102.0
4- 4-40	101.0	101.0
4- 5-40	101.8	101.2
4- 6-40	100.2	100.0

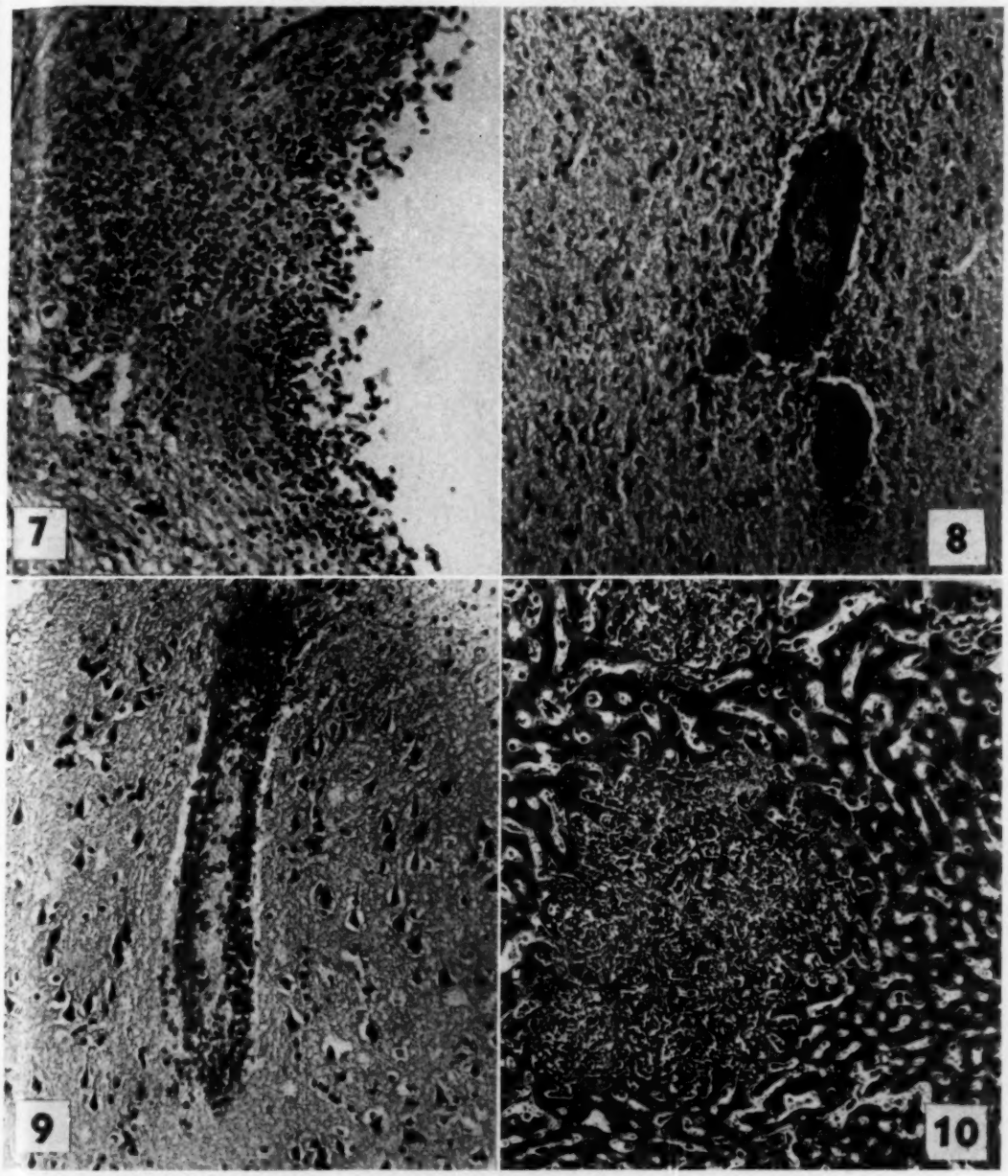
These two pigs made an uneventful recovery. They were kept under observation for some time and appeared to be in normal condition.

Inoculation of Bovine Listerella in Sheep

Intracranial Inoculation. — Three sheep were inoculated intracranially with 1 cc. of a suspension of culture 979 on March 28. The initial reaction was severe in each inoculated animal. Sheep 1 and 2 died on March 30. Sheep 3 died on April 5. The temperature reactions were as follows:

Date	*Sheep 1*	*Sheep 2*	*Sheep 3*
3-29-40	107.0	103.0	107.0
3-30-40	Died	Died	105.8
3-31-40			106.2
4- 1-40			107.0
4- 2-40			105.2
4- 3-40			106.0
4- 4-40			104.0
4- 5-40			Died

Postmortem examination revealed similar pathologic changes in the three sheep. The eyes were clear and some discharge from the nasal cavities was observed. The livers were slightly enlarged and yellowish. The hearts showed some evidence of degenerative changes, especially in sheep 3. The brain tissue was congested and edematous in the meningeal and submeningeal regions. There was some edema in the cerebral hemispheres and in the region of the pons. Considerable hemorrhage was observed in the base of the brain in sheep 3. Cultures from the brain tissue were positive. Cultures made from the liver, spleen, kidneys, lymph nodes and heart blood were negative.



7) Typical reaction in ventricles and cerebral ducts of sheep. Experimental infection (x 150).
8) Brain, showing perivascular cuffing from intracranially injected chicken (x 150).
9) Perivascular response in brain of experimentally injected rabbit (x 150).
10) Liver of intravenously injected rabbit, showing foci of infiltration and necrosis-containing organisms (x 150).

The histopathologic changes encountered in sheep followed the same general pattern as that of swine. The brain of sheep 3, which died seven days following inoculation, showed the most advanced cellular reactions. The perivascular cuffings were widely distributed. Focal monocytic infiltrations were numerous and hemorrhages also were found. Advanced meningitis was noted. Considerable destruction of epen-

dymal cells and subependymal monocytic infiltrations were observed. Hemorrhages also were present (figs. 6 and 7).

Intravenous Inoculation. — Two sheep were inoculated intravenously with 5 cc. of a suspension of culture 979. The initial reaction was severe and was accompanied by a rise in temperature. Both sheep appeared depressed and failed to eat or drink until the temperature reaction subsided three days following inoculation. Both recovered.

Inoculation of Bovine Listerella in Chickens

Intracranial Inoculation.—On March 28, three native White Leghorn hens were inoculated intracranially with 0.1 cc. of a suspension of culture 979 in the same concentration previously used for experimental inoculation. Chicken 1 died on March 29, chicken 2 on March 30 and chicken 3 recovered after showing a mild reaction for several days. The pathologic lesions found in the two fatal cases were similar. The brain tissue was soft and congested. The liver was slightly congested, light mahogany in color and friable. The spleen appeared slightly enlarged. The heart showed evidence of degenerative changes. Cultures made from the brain were positive. Cultures made from the liver and heart blood were negative. The histopathologic changes in the brain were widely distributed and severe. The perivascular involvement was pronounced (fig. 8). The heart showed slight degenerative changes and isolated infiltrating cells in the interstitial tissues were observed. The severe changes associated with field cases of avian listerellosis were absent.

Intravenous Inoculation. — Three White Leghorn hens were inoculated intravenously with 0.5 cc. of a suspension of culture 979. No apparent reaction was observed in these birds as the result of the inoculation. They were kept under observation for several weeks. They were destroyed and examined, but pathologic lesions could not be detected. Cultures made from the various organs were negative.

Inoculation of Bovine Listerella in Rabbits

Intracranial Inoculation. — Two rabbits, each of which received 0.1 cc. of a suspension of culture 979, died in less than 24 hours. Listerella organisms were obtained from the brains, but not from the livers. Severe microscopic changes were present in the brain tissue (fig. 9).

Intravenous Inoculation. — Two rabbits were inoculated intravenously with 1 cc. of a suspension of culture 979. These rabbits succumbed two and four days, respectively, following inoculation. From rabbit 1, Listerella cultures were obtained from the heart blood, spleen and liver, but not from the brain and kidneys. Cultures from the brain, heart blood, liver and spleen of rabbit 2 were positive whereas those from the kidneys remained sterile.

Histopathologic study of the brains of the intravenously inoculated rabbits revealed only a slight general reaction with complete absence of the changes usually associated with intracranial injections. The heart showed cloudy swelling, hemorrhages and a slight cellular infiltration. The liver showed retrograde changes, congestion and interstitial cellular infiltration as well as focal necrosis. Listerella organisms were demonstrated in these foci (fig. 10).

Serologic Data

The organisms isolated from field cases of bovine origin appear to be identical to our ovine and porcine strains. They are short rods, rather uniform in size, appearing singly or in short chains (fig. 1). They are gram-positive when stained by Hucker's modified gram stain. Because of the similarity of these organisms originating from these different species of domesticated animals, it seemed possible that they might possess similar or identical antigenic properties. Investigations were made to determine cross-agglutinating properties of the bovine immune serum against its

homologous antigen and that of the ovine and porcine strains. Antigens were made and standardized from ovine culture 910, bovine culture 979 and porcine culture 1079. The blood serums from calves 1 to 7 were taken periodically through the period of reaction and during the period of observation subsequent to various inoculations with bovine culture 984 and 1054. The agglutination reactions are indicated and summarized in table II.

In most instances the serums obtained from the blood of the calves inoculated with the bovine strain of Listerella agglutinated the antigens prepared from ovine, bovine and porcine strains. The serums from calves 3 and 6 failed to show any positive reaction on antigen 910. Likewise calf 6 failed to show a positive reaction on antigen 1079. The calves which were inoculated subcutaneously and intracranially showed the strongest reactions. As previously reported in the case of ovine and porcine infections, the agglutination reactions with standard antigens are so variable and inconsistent that this procedure is of questionable value for diagnostic purposes.

SUMMARY

A number of outbreaks of an infectious disease in cattle manifesting central nervous disturbances were found to be caused by a small, motile, gram-positive organism belonging to the genus Listerella.

Experiments conducted on the transmission of this disease indicate that cattle are far more resistant than either sheep or swine.

The organism apparently loses its pathogenicity after repeated cultivation on artificial media. The virulence may be restored by serial passage through a series of laboratory animals.

Infections of various degrees of intensity were reproduced under experimental condition. Fatal cases were obtained by intracranial inoculations in cattle. Intravenous inoculation in cattle resulted in a severe reaction of relatively short duration followed by complete recovery.

Intracranial inoculation of the bovine strain proved fatal to the experimental swine. The intravenous inoculations produced less severe reactions followed by recovery.

The disease transmitted to sheep by in-

TABLE II—Agglutination Reactions

Antigen	Calf No.	Culture No.	Mode of Inoculation	Dilutions 1:25	1:50	1:100	1:200	1:400
910	1	984	Intracranial	+++	++	+	—	—
	2	1054	Intracranial	+++	++	+	—	—
	3	984	Intravenous	—	—	—	—	—
	4	1054	Intravenous	+	—	—	—	—
	5	984	Oral	+++	+++	+	—	—
	6	1054	Oral	—	—	—	—	—
	7	1054	Subcutaneous	++++	+++	++	+	—
979	1			+++	++	+	—	—
	2			++	+	±	—	—
	3			+	—	—	—	—
	4			++	±	—	—	—
	5			+++	++	+	—	—
	6			++	+	—	—	—
	7			++++	+++	+	—	—
1079	1			+++	++	+	—	—
	2			++	+	—	—	—
	3			++	+	—	—	—
	4			++	±	—	—	—
	5			++	+	—	—	—
	6			—	—	—	—	—
	7			+++	++	+	—	—

tracranial inoculation terminated fatally. The sheep inoculated intravenously experienced a severe reaction accompanied by a rise in temperature which was of short duration followed by complete recovery.

Fatal infections were secured by intracranial inoculations in chickens. Intravenous inoculations in chickens produced no apparent reaction.

The bovine strain of this organism is similar morphologically to that isolated from sheep and swine and possesses similar staining properties as well as cultural characteristics. Inoculation of animals with the cultures of the bovine strains produced agglutinins in the serums of most of them. These serums agglutinated antigens of both ovine and porcine strains. The reactions, however, were so variable and inconsistent that they can not be depended upon as a routine laboratory procedure.

Epithelial Tumors of Horses*

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THIS PAPER INCLUDES a variety of information concerning epithelial tumors of horses which were diagnosed by the authors for the veterinary clinic staff of Iowa State College from 1920 to 1940. In all, there were 73 benign and malignant squamous-cell and gland-cell tumors. The number in each category is: papillomas 7, squamous-cell carcinomas 54, adenomas 2 and adenocarcinomas 10.

PAPILLOMAS

Because of the ease of clinical diagnosis and the absence of alarming effects associated with most papillomas, they have seldom been submitted for histologic examination. The seven that have been studied were removed from the following sites: forehead 1, penis 2, prepuce 1, clitoris 1 and frog 2.

With only a glance at this list of locations one is struck by the fact that the clinician, for special reasons, was interested in knowing the seriousness of the tumor in each case before attempting surgical removal. The least important tumor in this group from the clinician's point of view was the papilloma of the forehead. It was examined principally to satisfy the surgeon's curiosity because the growth was on the face of a very young colt and measured 6 x 5 x 2 cm.

SQUAMOUS-CELL CARCINOMAS

The most impressive feature of the 54 squamous-cell carcinomas examined was the preponderance of eye incidence. The eye, principally the membrana nictitans, was the location 35 times, and all other parts of the body only 19 times. These figures confirm the usual statement made concerning the prevalence of eye carcinomas in horses.

The 35 ocular squamous-cell carcinomas in most cases, as just stated, had their location in the nictitating membrane. At the time of removal their period of growth had been from two to three weeks up to six years. The majority of them were of a few months' duration, often less than six months. Many were removed within a few weeks after their appearance; thus their size was relatively small (fig. 1). Some, however, were allowed to reach a size up to 10 cm. in diameter. As would be expected, those tumors characterized by rapid growth showed histologically a more embryonic type of epithelium and greater numbers of mitotic figures.

The owners of seven of the horses claimed that the development of the tumors was associated with previous injury to the eye. Squamous-cell carcinoma of the membrana nictitans apparently does not metastasize readily because metastasis occurred in only one of the 35 animals. In this case

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there was no metastasis until a recurrence of the tumor appeared two years after its apparent surgical removal. Even then a year passed before the animal was brought to the clinic, during which time the carcinoma had infiltrated the masseter muscle down to the ventral and posterior borders of the mandibular ramus, and had only recently mestastasized to the mandibular and anterior cervical lymph nodes.

For 24 of the 35 horses having squamous-cell carcinoma of the eye, the age was recorded. It averaged 8 years. Thirteen of the animals were in the age group 7 to 10 years. This tumor did not occur in any patient less than 4 years old.

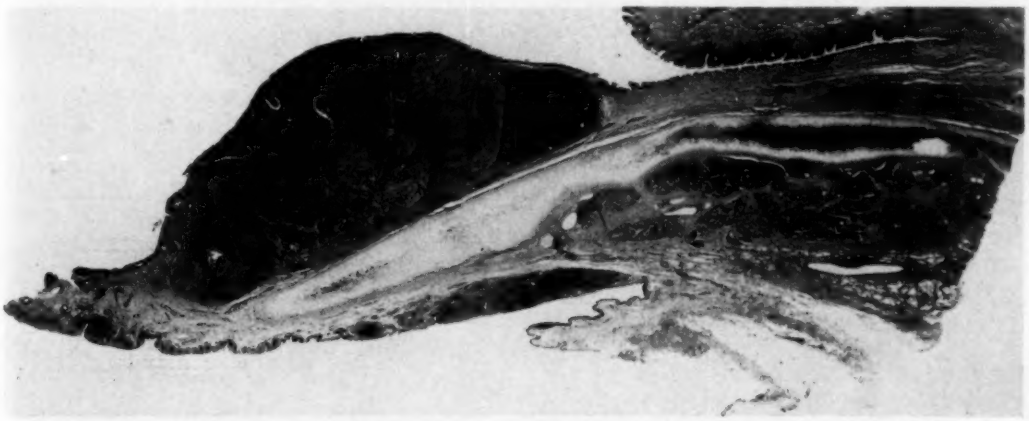

Fig. 1. A recent squamous-cell carcinoma of the membrana nictitans of a 10-year-old mare. The nodule was 8 mm. in diameter and extended down to the cartilage. The surface of the tumor was pinkish yellow and slightly granular (x 6).

For 42 of the 54 horses the age was recorded. Eighteen were geldings or stallions and 24 were mares. These figures are probably not significant.

The locations of the 19 squamous-cell carcinomas other than those occurring in the eye were:

Head and neck: maxillary sinus 1, oral cavity 3, chin 1, throat region 3.

External genitals: glans penis 2, prepuce 4, clitoris 1, vulva 2.

Leg: on the skin above the hock 1.

Tail: on the skin near the base 1.

Head and Neck.—The tumor of the maxillary sinus was from an 11-year-old horse. Metastasis had occurred to the mandibular lymph node. The three carcinomas of the mouth appeared to originate on the gingival mucosa around the base of the teeth. From one of these tumors metastasis had occurred to a cervical lymph node. The chin carcinoma was removed from a 12-year-old mare. The owner associated the tumor with a severe injury suffered when the horse was young. The three neoplasms of the throat region were located just behind one of the posterior borders of the mandibular ramus. Two of them had metastasized to the mandibular lymph nodes. One of these primaries was the largest tumor in this whole series of carcinomas. It extended downward from the base of an ear for a distance of 30 cm. and measured 10 cm. in diameter. It weighed aproximately 2.15 kg.

External Genitals.—There was nothing unusual about the squamous-cell carcinomas of the penis and prepuce except that metastasis apparently had not occurred even when the tumors were exceptionally large. One, however, on the prepuce of a 12-year-old gelding recurred four years after apparent surgical removal. It had been developing about three months at the time of the first removal, but had only become about 3 cm. in diameter. The same statement could not be made with reference to the vulval carcinomas because one of the two metastasized to the lungs. It occurred in a 20-year-old mare. The other vulval carcinoma attracted attention because of its

origin. The epithelial cells forming it were derived from the hair matrix. It could be designated, therefore, as a hair-matrix carcinoma. Four and one-half months after its surgical removal it showed no indication of recurrence.

Rear Leg.—A cutaneous growth 10 x 8 x 10 cm. in size was removed from the leg dorsoposteriorly to the hock. It had been present two years. Histologically it was a highly malignant squamous-cell carcinoma developing at the edge of a fibrosarcoma.

Tail.—From the standpoint of histogenesis a carcinoma from the tail of a 14-year-old gelding was of especial interest. It had been developing about four years and an attempt had been made to remove it by means of caustics a few months before the animal was brought to the clinic. By this time the tumor measured 15 x 10 x 10 cm. It was attached to the base of the tail by a pedicle 5 cm. in thickness. Histologically the tumor was derived from cells having more the characteristics of basal epidermal cells than of squamous cells.

ADENOMAS

The incidence of adenomas in horses in our clinic was exceptionally low. This tumor was encountered only twice: once in the medulla of a kidney and once in a mammary gland.

ADENOCARCINOMAS

Adenocarcinomas were obtained from ten horses whose ages were recorded in only four instances. One was 5 years old, two were 11 and one was 12. The tumors were located as follows: eyelids 3, oral cavity and lower jaw 1, guttural pouch 1, thyroid gland 3 and mammary gland 2.

Judging from the experience in our clinic the eyelid adenocarcinoma of horses is more malignant than the squamous-cell carcinoma of the membrana nictitans. For instance, one adenocarcinoma developed on the upper lid of an 11-year-old mare. The eye reportedly had been "injured" when the mare was 2 years old, but the wound healed. Six months prior to admission of the patient a veterinarian removed part of the lid together with the tumor, but recurrence took place within six months. The neoplasm was "removed" again, but within three weeks it showed indications of even more rapid growth than before the second operation. The horse was used in a class for surgical practice and then examined *post mortem*. No metastasis was apparent. In another horse of unrecorded age an adenocarcinoma involving both eyelids recurred twice after apparent surgical removal. Metastasis occurred to the parotid lymph node.

The adenocarcinoma of the mouth and lower jaw developed at the site of a fracture of the mandible in a 12-year-old gelding. The tumor was most prominent in the mouth, where it attained a size of 25 x 10 x 10 cm. Growth had been very rapid, especially within the week prior to the operation.

It was soft, for the most part covered with epithelium, and bled easily when traumatized. Histologically it was a scirrhous carcinoma with islands of very embryonic, rapidly growing cells which had a glandular arrangement.

One of the mammary gland carcinomas was a recurrence of a tumor that had been "removed" a year previously. It was about 8 or 10 cm. in diameter and was cystic. The other mammary gland carcinoma came from an 11-year-old mare in which the tumor had been developing for two months. The condition of the mare indicated metastasis. This diagnosis was substantiated by a necropsy which showed metastatic foci in the supramammary and iliac lymph nodes, lungs and contiguous lymph nodes.

SUMMARY

Of the 73 epithelial tumors of horses which form the basis of this report, 7 were papillomas, 54 squamous-cell carcinomas, 2 adenomas and 10 adenocarcinomas.

Only papillomas located where they interfered with body function and that resembled malignant tumors were submitted by the clinician to the pathologist for diagnosis.

Of the 54 squamous-cell carcinomas 35 were removed from the eye, mostly from the membrana nictitans. Only one of the

35 metastasized. The average age of 24 horses with ocular squamous-cell carcinoma was 8 years.

The squamous-cell carcinomas occurred 18 times in geldings and stallions, 24 times in mares and 12 times in horses whose sex was not noted.

The 19 squamous-cell carcinomas located in other parts of the body were mostly removed from the head and external genitals. Squamous-cell carcinomas of the external genitals of male animals did not metastasize and only one is known to have recurred. One of the 3 in mares metastasized.

Judging from the data collected at our clinic, adenomas and adenocarcinomas are relatively rare in horses. Two of the three adenocarcinomas of the eyelid metastasized, which could lead one to assume that adenocarcinoma of the eyelid of horses is more malignant than squamous-cell carcinoma of the nictitating membrane.

Most of the malignant epithelial tumors of horses appeared when the animals were 7 to 12 years old.

Neutralization Studies of the Viruses of Influenza A, Influenza B and Swine Influenza with Equine Influenza Convalescent Serums*

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THE MARKED SIMILARITY of the clinical manifestations of influenza in man and in horses has suggested to many authors the possibility of related etiologic factors in the two diseases. Early writers, such as Sisley,[1] Raymond[2] and Dieudonné,[3] attempted to determine if an etiologic relationship between the two diseases existed on the basis of epidemiologic observations. In 1918, Bemelmans[4] concluded that the diseases are identical, except for minor species differences. In 1927, he further discussed this theory.[5] The occurrence of human influenza in swine, as reported by Shope,[6] and the close relationship of swine and human influenza is also interesting in this connection.

These observations suggested the need for more information on the relationship of the two diseases, each called influenza, in the horse and in man. The occurrence of a severe outbreak of equine influenza in Army horses and mules during 1939 and 1940 afforded exceptional opportunities for study. At Army remount depots large numbers of young susceptible animals are concentrated. The occurrence of equine influenza among these animals is common. Over 400 samples of preinfection and convalescent serums were collected at the Front Royal Remount Depot. Not all of these serums were tested, as subsequent findings did not seem to warrant it.

MATERIALS AND METHODS

Virus.—Glycerolated infective mouse lung material was used as the virus. All viruses*

*From the Veterinary Research Laboratory, Front Royal Remount Depot.

[1]Sisley, R.: Is influenza in man and animals aetiologically distinct? Trans. Epidemiol. Soc., xi (1891-92), pp. 60-69.

[2]Raymond, F.: Is influenza etiologically distinct in man and animals? Brit. Med. J., i (1892), pp. 681-682.

[3]Dieudonné: Zur Aetiologie der Influenza beim Pferde und ihrem Causalzusammenhang mit der Pneumonie des Menschen. Deut. mil-ärtzl. Zeit., xxi (1892), pp. 99-108.

[4]Bemelmans, E.: A comparative study of human grippe and contagious pleuropneumonia of the horse (equine grippe). (Title trans.) Rev. Gén. Méd. Vét., xxx (1921), p. 377.

[5]*Ibid.*: Van de influenza (griep) by den mensch en van de eigenlijke influenza (de zoogenaamde borstziekte) van het paard. Geneesk. Gids, 's Gravenh., v (1927), pp. 173-183.

*The authors are grateful to Drs. R. E. Shope, Thomas Francis Jr. and Thomas P. Magill for the mouse-adapted viruses used. Dr. Shope also supplied us with various antiserums used in confirming the identity of some of the viruses used.

[6]Shope, R. E.: Serological evidence for the occurrence of infection with human influenza virus in swine. J. Exp. Med., lxvii (May 1, 1938), pp. 739-748.

were adapted to mice and, with the exception of the TM virus, were regularly lethal to mice. Weighed mouse lung was triturated in crystalline alundum and diluted in nutrient broth initially to 10 per cent. This suspension was then centrifugalized at slow speed for five minutes. The supernatant fluid was diluted further in broth to make the virus suspension. Prior to its mixture with the serum to be tested, the virus suspension, except in the case of the TM virus, contained between 100 and 1,000 mouse m.l.d. per 0.05 cc. As it was desired to recognize even small amounts of antibody, the lower limit was most frequently approximated. Virus titrations were done at the same time as each group of neutralization tests. The TM virus, being of lower virulence for mice, was used in the concentration of about 10 m.l.d. It was found necessary to add normal (preinfection) horse serum to the broth used in suspending the virus in order to get a uniformly lethal suspension. The 50 per cent mortality end-points of Reed and Muench[7] were used throughout these experiments in determining the virus titer.

The viruses used in this work were Shope's strain 15 of swine influenza; PR-8, Melbourne, and WS strains of influenza A; and the TM strain of influenza B virus.

Antiserums.—Antiserums for the repeated identification of the viruses were prepared by the intraperitoneal inoculation of rabbits with a heavy suspension of infected mouse lung. The serum was collected 13 days later. Horse serums were collected aseptically prior to the appearance of symptoms of equine influenza and again at varying intervals after the thirteenth day following initiation of symptoms. The identity of some of the viruses was further checked by neutralization with specific antiserums prepared from swine.

Technic of Neutralization Tests.—Each serum to be tested was placed in 0.5-cc. amounts in a sterile Wassermann tube. An equal amount (0.5 cc.) of a suspension of infective mouse lung in broth was added to each tube. The tubes were shaken and placed in a refrigerator at 6 C. for two hours. At the end of that time 0.05 cc. of each mixture was inoculated intranasally into each of four white mice under light ether anesthesia. The mice weighed approximately 10 Gm. each. A sterile 0.5-cc. glass syringe fitted with a 23-gauge needle was used for each inoculation. All mice dying within ten days were autopsied and the lungs examined. At the end of ten days all surviving mice were destroyed, similarly examined and the results recorded.

[7]Reed, L. J., and Muench, H.: A simple method of estimating fifty per cent endpoints. Am. J. Hyg., xxvii (1938), pp. 493-497.

INTERPRETATION OF RESULTS

The survival of two or more of the four mice inoculated was interpreted to mean that the virus was neutralized. Usually all four mice inoculated with one serum either survived or died during the experiment. In the case of the few horse serums that partially neutralized the virus used, further tests were performed to indicate the approximate amount of antibody present. In each lot of tests this virus used was neutralized by its homologous antiserum. This eliminated the possibility of pneumonia-producing mouse viruses being present in any virus suspension.

DISCUSSION

Representative cases were selected from animals that had been shipped from various parts of the United States. The serums of these animals were tested for neutralizing antibodies against several mouse-adapted viruses. The results of these tests are given in table I. Only two preinfection serums are included in this table, but the results are representative of all others tested.

It will be noted in table I that, in general, the rabbit antiserums were specific in neutralizing their homologous virus. However, the antiserum prepared against the Melbourne strain of virus neutralized all three influenza A strains, but not the swine or the TM viruses. The swine-influenza antiserum neutralized the WS strain of human virus as well as its homologous virus. However, this antiserum possessed an endpoint of 1:5 against the WS strain and above 1:20 against the swine virus. None of the antiserums prepared against the influenza A viruses neutralized either the swine or the TM viruses. These findings are in general agreement with those of Magill and Francis,[8] who found that influenza A antiserums did not neutralize the swine virus, but that the swine-virus antiserums could neutralize influenza A virus.

It is readily apparent from table I that, in general, equine influenza convalescent serums have no neutralizing ability against

[8]Magill, T. P., and Francis, T. Jr.: Antigenic differences in strains of human influenza virus. Proc. Soc. Exp. Biol. & Med., xxxvi (1936), pp. 463-466.

TABLE I—Results of Neutralization Tests

Serum No.	Convalescent from	Virus Strain Tested Against				
		Influenza A			Swine	Influenza B
		PR-8	Melbourne	W. S.	Shope Strain 15	TM
R 459	PR-8	0, 0, 0, 0	5, 6, 7, 8	9, +++, ++, ++	4, 5, 5, 6	5, 6, 7, 8
R 452	Melbourne	0, 0, 0, 0	0, 0, 0, 0	+, +, 0, 0	3, 4, 4, 4	5, 5, 6, 7
R 431	W. S.	5, 6, 7, 7	5, 5, 6, 6	0, 0, 0, 0	5, 5, 8, 8	4, 5, 5, 7
R 433	Swine	7, 7, 9, 9	5, 5, 7, 7	0, 0, 0, 0	0, 0, 0, 0	5, 5, 6, 7
R 455	TM	3, 3, 4, 4	6, 7, 7, ++++	10, ++, +++, ++	4, 4, 4, 4	+, 0, 0, 0
R 446	Normal rabbit serum	5, 5, 5, 5	5, 5, 5, 6	6, 8, 10, 10	4, 4, 4, 5	5, 6, 7, 7
H 152	Preinfection (see 398)	4, 5, 5, 5	5, 6, 8, 0	4, 6, 6, 6	5, 5, 6, +++	4, 5, 5, 6
H 398	E. infl. (17 days)	4, 5, 5, 7	5, 6, 7, 9	6, 6, 8, ++	3, 5, 5, 6	3, 5, 7, 9
H 164	Preinfection (see 166)	5, 5, 5, ++++	6, 6, 6, ++++	8, 10, +++, 0	4, 4, 4, 5	5, 6, 7, X
H 166	E. infl. (14 days)	4, 5, 5, 5	4, 4, 5, 5	5, 6, 7, 7	6, 9, 9, ++	5, 5, 5, 8
H 3	E. infl. (21 days)	6, 6, 6, 6	5, 5, 6, +	5, 5, 5, 6	6, 7, 7, 8	7, 7, 8, 8
H 6	E. infl. (19 days)	4, 5, 6, 7	5, 5, 6, 7	5, 6, 6, 7	7, 9, 9, +++	5, 5, 6, 7
H 7	E. infl. (13 days)	5, 6, 6, +	5, 6, 6, 7	6, 6, 7, 7	6, 6, 7, 8	4, 5, 5, 5
H 8	E. infl. (21 days)	5, 5, 5, 6	5, 5, 5, 8	2, 4, 5, 7	10, ++, +, 0	6, 9, 9, 0
H 98	E. infl. (33 days)	4, 5, 5, +	6, 6, 6, 7	5, 6, 6, +	4, 4, 5, 6	4, 5, 6, 6
H 102	E. infl. (33 days)	3, 4, 6, 9	5, 5, 6, 7	5, 5, 9, 10	2, 4, 4, 5	4, 6, 6, 8
H 105	E. infl. (21 days)	4, 5, 5, 7	6, 8, 8, 10	5, 5, +++, 0	4, 4, 4, 6	7, 8, 10, 10
H 118	E. infl. (22 days)	4, 6, 6, 9	5, 5, +++, ++++	5, 5, 5, 7	4, 5, 6, 6	4, 5, 5, 6
H 157	E. infl. (80 days)	6, 6, 6, 0	4, 4, 4, 5	5, 5, 5, 6	7, 8, 8, 9	4, 4, 6, 7
H 158	E. infl. (84 days)	1N, 5, 7, 7	5, 5, 6, 7	5, 7, 7, 8	7, 8, 8, ++++	5, 5, 5, 5
H 191	E. infl. (30 days)	5, 6, 6, 7	6, 8, 8, 8	5, 5, 5, 9	4, 5, 5, 5	4, 5, 5, 5
H 207	E. infl. (21 days)	5, 6, 7, +	4, 4, 5, 0	5, 5, 5, 9	4, 5, 6, 6	5, 6, 7, 8
H 223	E. infl. (? days)	3, 3, 4, 4	5, 6, 7, 8	4, 5, 6, 6	7, 8, 8, 9	4, 5, 5, 6
H 319	E. infl. (105 days)	6, 6, 6, ++	4, 4, 6, 6	5, 5, 5, 6	4, 5, 6, 7	4, 5, 5, 8
H 334	E. infl. (16 days)	4, 5, 5, 6	5, 5, 6, 6	5, 5, 6, 6	4, 5, 5, 6	4, 5, 7, 8
H 367	E. infl. (17 days)	5, 5, 5, 6	6, 8, 8, 10	5, 5, 5, 6	6, 7, 7, 7	6, 6, 7, 8
H 400	E. infl. (20 days)	5, 6, 6, 6	3, 3, 5, ++++	7, 7, 9, ++++	2, 2, 6, 10	5, 5, 6, 7
H 401	E. infl. (21 days)	6, 6, 6, 9	5, 5, 5, 6	5, 6, 6, 7	7, 8, 8, 8	4, 5, 6, 7

TABLE II—Tested Against Swine-Influenza Virus

Virus Used	Dilutions of Serum No. 8					
	Undiluted	1:1	1:2	1:4	1:8	1:16
Swine influenza (1,000 m.l.d.)	6, ++, +++, ++	7, 8, ++, +++	7, 8, 9, 9	6, 6, 6, 9	5, 5, 6, 7	5, 5, 6, 6

TABLE III

Serum No.	Dilutions of Serum					Virus Used
	Undiluted	1:2	1:5	1:10	1:20	
H 400	7, 7, 9, ++++	7, 7, 8, 8	5, 6, 7, 8	.....	.....	W. S.
R 431 (anti W. S.)	9E, 0, 0, 0	0, 0, 0, 0	0, 0, 0, ++	8, +++, ++, +++	7, 7, 7, 10	W. S.

Plus marks indicate the degree of pneumonia in each mouse destroyed on the tenth day following inoculation.

O = No lesions found at autopsy on the tenth day.

X = Three mice inoculated.

N = Mouse died with no pulmonary lesions.

Each numeral indicates the day of death from typical pneumonia of one mouse.

the viruses tested. A few exceptions do appear, however. Serum No. 8 partially neutralized the swine-influenza virus. Dilutions of this serum were tested against the swine virus to determine the approximate amount of antibody present. This is shown in table II.

Serum 8 was taken from a horse purchased at Front Royal, Va., on November 19, 1940. This animal became sick with equine influenza on December 5. Serum was collected 21 days later. Unfortunately, preinfection serum was not collected from this horse. However, serum collected on the thirty-seventh day of convalescence from this horse was tested against the swine virus. This serum specimen had a neutralizing ability similar to that of the sample taken on the twenty-first day of convalescence. Since this animal was sick in the same lot and at the same time as many of the other animals tested, it does not seem likely that the neutralizing capacity of its serum is a result of equine influenza. Whether this animal was previously exposed to swine influenza is not known.

Serum 400 appeared to have an inhibitory effect upon the WS and Melbourne strains of virus. When compared quantitatively with rabbit antiserums prepared against these two virus strains, this effect did not seem significant. This is illustrated in table III.

CONCLUSIONS

Serums from horses convalescent from equine influenza were found to have no significant increase in neutralizing capacity against the WS, Melbourne and PR-8 strains of influenza A, swine-influenza virus, or the TM strain of influenza B virus.

Transmission of Equine Infectious Anemia by the Stablefly, Stomoxys Calcitrans, the Horsefly, Tabanus Sulcifrons (Macquart), and by Injection of Minute Amounts of Virus*

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ALTHOUGH EXPERIMENTAL evidence had been reported to indicate that infectious anemia may be transmitted when infected and susceptible horses are kept in prolonged intimate contact, the relatively fewer cases of apparent transmission observed under these conditions when biting flies were excluded and the difficulty encountered in readily transmitting the disease by feeding contaminated material led many of the earlier investigators to suspect that blood-sucking or biting pests of horses may play an important rôle in the natural transmission of the disease.

REVIEW OF LITERATURE

Observations on the occurrence of natural transmission have indicated that infectious anemia is essentially a pasture disease with a seasonal occurrence closely corresponding with the fly season. The ease with which it can be transmitted experimentally by subcutaneous injections of minute quantities of the virus suggests that one or more of a number of agents might act as vectors under natural conditions. The Japanese Commission[1] was unable to transmit the disease by ticks (Ixodidae) from infected to susceptible horses; it was also unable to show that sand flies (Simulium) transmit the infection. Francis and Marsteller[2] were unable to transmit the disease by transferring to a susceptible horse several hundred young ticks (*Boophilus annulatus*) from the eggs of 20 mature females collected from an infected horse. Semenov and Kozlova[3] reported transmission with the biting type of horse lice (*Trichodectes equi*) when 300 lice were transferred from an infected colt and were placed on a susceptible animal which developed the disease 32 days later. These authors also presented evidence that a suspension of eggs from the lice present on infected horses contained the virus. Lührs[4] failed to transmit the disease with the blood-sucking louse, *Haematopinus asini*, and was unable to demonstrate the presence of the virus in lice removed from infected horses. Stein, Osteen and Mott[5] obtained negative results when 45 *H. asini* were transferred from an acute case of the disease to a susceptible horse.

The question of whether bot flies (*Gastrophilus equini*) may be a factor in the transmission of the disease was investigated by Favero,[6] Van Es and Schalk,[7] Scott,[8] Flocken,[9] Stein, Lucker, Osteen and Gochenour,[9] and others, all of whom failed to show any causal relationship between Gastrophilus larvae and infectious anemia. Lührs,[4] however, reported transmission of the disease by subcutaneous injection of an extract of Gastrophilus larvae.

The ability of mosquitoes to transmit the

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*This investigation was a coöperative project of the Zoölogical and Pathological Divisions of the Bureau of Animal Industry, U. S. Department of Agriculture, carried out during the summer of 1941 at the Animal Disease Station, Beltsville Research Center, Beltsville, Md.

[1]Japanese Commission (Horse Administration Bureau, Tokyo, 1914). Report on the results obtained by the special committee for investigation of infectious anemia of the horse. Vet. J., lxx (Dec. 1914), pp. 604-627.

[2]Francis, M., and Marsteller, R. P.: Some recent experiments on infectious anemia of the horse. Am. Vet. Rev., xxxix (May 1911), pp. 132-147.

[3]Semenov, M. A., and Kozlova, E. S.: Lice as vectors of equine infectious anemia (title trans.). Travaux Inst. Méd. Vét. Exp. URSS, No. 112 (French summary, 1936), pp. 38-45.

[4]Lührs: Die ansteckende Blutarmut der Pferde. Zeit. f. Veterinärk., xxxi (1919), Nos. 10-12.

[5]Stein, C. D., Osteen, O. L., and Mott, L. O.: A résumé of studies and observations on infectious anemia, or swamp fever of horses, carried on by the Bureau of Animal Industry from 1935 to 1940 (Mimeo., Pathological Div., BAI, USDA, Washington, D. C., Feb. 1941); report of the chief of the Bureau of Animal Industry (1938).

[6]Favero, F.: Larve di Gastrophilus equi ed haemorrhoidalis e tifo-anemia infettiva del cavallo. Unova Ercolani, xxi (1916), pp. 4-7, 17-21.

[7]Van Es and Schalk: Notes on parasitic anaphylaxis and allergy. North Dakota Agr. Exp. Sta. Bul. 125 (1917).

[8]Scott, J. W.: Insect transmission of swamp fever or infectious anemia of horses. Wyoming Agr. Exp. Sta. Bul. 133 (June 1922).

[9]Stein, C. D., Lucker, J. T., Osteen, O. L., and Gochenour, W. S.: Studies on the possible rôle of endoparasites in the transmission of infectious anemia. J.A.V.M.A., xcv (Nov. 1939), pp. 536-541.

*Unpublished results of experiments performed by Flocken in the Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture.

disease has been tested to a limited extent. Lührs,[4] working with Anopheles mosquitoes captured in stables occupied by horses affected with infectious anemia, demonstrated that the virus was present in the stomach contents of the mosquitoes and that the disease could be transmitted to a susceptible horse by the bites of the mosquitoes; the presence of virus in the larvae from such mosquitoes could not be demonstrated. Scott[5] reported two unsuccessful attempts to transmit the disease with Aedes mosquitoes in Wyoming.

A number of workers have especially stressed the possibility that certain biting flies with interrupted feeding habits may be principally concerned with the spread of infectious anemia. By a series of outdoor penning experiments, the Japanese Commission[1] was able to demonstrate that the disease was easily transmitted where infected and susceptible horses were kept in close proximity with no actual contact with each other under conditions when there is free access of biting flies. However, transmission occurred with difficulty and only after prolonged intimate contact of infected and susceptible horses in screened pens. Although Stomoxys and several species of horseflies were prevalent in the pasture during the experiments, the Commission concluded that certain species of Chrysops and Tabanus were the real agents of transmission of the disease in Japan, since these flies appeared in greater numbers during the season when the disease increased in prevalence. In 1936, Rodionov[11] conducted a pasture experiment in which five affected horses were stationed 120 meters (36½ ft.) from three healthy horses. Since after a period of 43 days one of the susceptible horses showed symptoms of the disease, the insects, *Chrysozoma pluvialis*, *Tabanus masculacornus* and *T. lapponicus*, which were prevalent during the experiment were suggested as possible transmitters of the disease. Howard,[10] in coöperation with Flocken, a Bureau investigator, reported evidence that the infection may have been carried from one horse to another by the stablefly (Stomoxys), but negative results were obtained in an experiment involving a total of 71 bites by the horseflies, *T. lasiophthalmus* and *T. affinis*. Lührs,[4] in a series of experiments carried out with stableflies collected in stables occupied by horses affected with infectious anemia, demonstrated by horse inoculation tests that the stomach and intestinal contents of these insects contained the virus, but several attempts to transmit the disease by allowing such flies to bite susceptible animals were negative. The author also demonstrated the presence of virus in the stomach and intestinal contents of Chrysops and other tabanids and concluded that the virus is not destroyed by the stomach and intestinal juices of such flies. Zenkner *et al*[12] collected 80 stableflies (Stomoxys) while the insects were feeding on a naturally infected horse and produced a fatal case of the disease in a susceptible horse by subcutaneous inoculation with the mouth parts of the flies.

The most convincing and direct evidence that biting flies may be involved in the natural transmission of infectious anemia is found in the work of Scott.[5] This author conducted a series of experiments involving both the penning of various insects with infected and susceptible horses and the direct manual transfer of the insects in a manner to simulate interrupted feedings of the insects on infected and susceptible horses. He reported positive results in experimental transmission of the disease with both *Stomoxys calcitrans* and the horsefly, *T. septentrionalis* (Loew).

Aside from the work of Scott, the experimental evidence for the transmission of the disease by biting flies is, for the most part, suggestive but inconclusive.

To clarify further the probable rôle of the stablefly and horseflies as possible vectors of infectious anemia, the experiments recorded in this paper were designed. Special attention was given to the virulency of the virus and the incubation period and the course of the disease in both the "source" animals inoculated by either subcutaneous or intravenous injection of filtered virus and in horses experimentally infected by flies which previously fed on the "source" animals.

MATERIAL AND METHODS

The strain of virus used for infecting the "source" animals in these experiments was obtained from blood samples forwarded to the Pathological Division by Dr. F. H. Melvin, in charge of the Bureau of Animal Industry field station at Cheyenne, Wyo. The samples were collected by Dr. L. A. Marshall, a Bureau field veterinarian, from subacute cases in an infectious anemia outbreak in Wyoming during March 1941.

This strain of virus was unusualy virulent. Small amounts on third passage produced the disease in the acute or subacute form after a short incubation period in susceptible horses.

Prior to the inauguration of the fly transmission experiments, a titration test was made on the strain of virus used for infecting host animals to determine its virulency and its power of infectivity in small amounts. Doses of 1 cc. each of the filtered serum collected

[10]Howard, C. W.: Insect transmission of infectious anemia of horses. J. Parasitol., iv (Dec. 1917), pp. 70-79.

[11]Rodionov, I M.: Dipterous insects as carriers of equine infectious anemia (title trans.). Med. Vet. Sovietique, No. 12 (1936), pp. 11-18.

[12]Zenkner, J., Kolodziejska, Helena, and Jastrzebski, D.: Equine infectious anemia (title trans.). Wojsk. Przegl. weteryn, ix (1938), pp. 5-61 (French summary).

TABLE I—Exposure and Titration Test on Wyoming Virus

Horse No.	Filtered Serum Wyoming Strain 1297 (2nd Passage)*			Date of First Reaction (1941)	Incubation Period (Days)	Form of Disease	Termination
	Date (1941)	Amount	Method				
1275	7–3	1 cc.	Intradermic	7–17	14	Acute	Died 28 days following exposure
1246	7–3	1 cc.	Intravenous	7–14	11	Acute	Died 25 days following exposure
1252	7–3	1 cc.	Intracranial	7–13	10	Acute	Died 27 days following exposure
1289	7–3	1 cc.	Subcutaneous	7–14	11	Acute	Died 40 days following exposure
1268	7–3	1 cc. of a 1:10 dil. in saline	Subcutaneous	7–15	12	Acute	Died 26 days following exposure
1274	7–3	1 cc. of a 1:1,000 dil. in saline	Subcutaneous	7–28	25	Acute	Moribund and destroyed 51 days following exposure

*Serum filtered through Mandler filter candles and tested for bacterial contamination before being administered.

from a horse infected with a second passage of the virus when administered to normal horses subcutaneously, intravenously, intradermically and intracranially produced an acute fatal form of the disease in from 10 to 14 days. One cc. of the serum in dilutions of 1:10 and 1:1,000 when administered subcutaneously likewise was infective for susceptible horses in 12 to 25 days. Data covering this experiment are shown in table I.

The host animals from which the flies received their infective feeds were horses inoculated by subcutaneous or intravenous injections of the highly virulent strain of infectious anemia filtered virus described above. Only acute cases resulting from the inoculations were used as "source" animals.

The susceptible horses used in these experiments were of the light type, approximately 900 to 1,100 lb., and were held under observation in screened isolation stables provided with concrete floors and screened vestibules. As far as can be determined, none of the normal horses had any previous contact with horses affected with infectious anemia. Temperatures on all of the test animals were taken and recorded at least twice daily. Following exposure to fly bites or with material suspected of containing virus, the course of the disease in all the animals was carefully studied and recorded.

Both the horseflies and stableflies used in the experiments were collected at the zoölogical station of the Beltsville Research Center, located about 2 miles from the Animal Disease Station, where the transmission experiments were made. The horseflies were collected in individual small cardboard boxes while they were feeding on cattle, and were used within two hours after they were captured. The stableflies were collected in a cowbarn and were kept for 24 to 48 hours in cages containing a dish of water with a few drops of honey to allow the insects sufficient time to digest any meals which they might have obtained previous to their capture and yet have sufficient food available to keep them alive.

Each experiment was conducted within a screened enclosure and special precautions were taken to exclude all possibilities of accidental transmission through direct contact of the infected and susceptible horses or through the agency of contaminated materials or unknown vectors. The transmission attempts with horseflies were conducted in a screened pen attached to the side of a stable, while that with the stableflies was conducted in a screened isolation stable.

Immediately following the transmission attempts with the horsefly, the experimental horses were moved into separate, screened isolation compartments connecting directly with the screened pen in which experiments were conducted, and the infected host animal was sprayed with a fly repellent and moved to an isolation stable.

In the stablefly transmission experiment, the normal experimental horse was moved to a screened isolation stable containing the infected "source" animal where the fly transmission attempts were conducted. At the conclusion of the fly transmission attempt, the normal experimental horse was thoroughly sprayed with fly repellent and quickly removed to a separate, screened isolation pen.

The procedure followed in conducting the insect transmission experiments was similar to that commonly used in investigations on various diseases. The horseflies were first allowed to begin feeding on the infected horse and were then quickly transported to the susceptible horse to resume feeding for a short time. The flies were then returned to the infected animal and this procedure was repeated until the flies refused to feed. The number of times each fly was transferred to and began feeding on the susceptible horse was recorded as the number of times the horse was bitten. A glass funnel and a piece of cardboard were

used in transporting the flies from animal to animal.

In the experiments involving the stablefly, about 20 of the flies were placed in each of a number of pint glass fruit jars covered with a fine mesh curtain material. Each jar was first placed with the cloth-covered end in contact with a clipped area on the infected horse. The behavior of the flies was observed and when it was found that certain of the insects were about two-thirds fully engorged, the approximate number of flies engaged in feeding was estimated. The jar was then gently lifted and placed on a clipped area on the back of the susceptible horse, where it was kept until all or nearly all flies refused to feed. Since the flies, when interrupted in their feedings, appeared to resume feeding after further opportunity was afforded and a larger number always fed on the susceptible horse, the number observed to be feeding on the infected horse was recorded as the approximate number which might be involved in transmitting the virus.

The horsefly mouth parts tested for the presence of virus were collected and handled as follows: The mouth parts from the 24 horseflies after being used in experiments 1 and 2 were removed and held at a temperature of 5 C. for four to five days. A saline suspension of the mouth parts was prepared for inoculation by grinding them in a sterile mortar, suspending in sterile normal saline solution and filtering through sterile gauze. A microscopic examination of a number of the mouth parts prior to trituration revealed that they were contaminated with small amounts of dried blood.

PROTOCOLS OF EXPERIMENTS

Table I shows exposure and titration tests on the Wyoming strain of virus used in the experiments. In this table are included horses 1246 and 1274, which were used as sources of infective material in the transmission attempts. Table II gives further information on the history of the virus, its source and its passage from horse to horse. For comparative purposes, table III gives summarized data on the character of the reaction obtained in experimental horses infected by fly bites and by other methods of exposure. Graph 1 gives a temperature record of source animal 1246, and graph 2 gives a temperature record of source animal 1274. Additional information and further elaboration on the tabulated data are given in the following protocols.

Experiment 1.—A normal horse, No. 1295, weighing approximately 1,050 lb., was bitten 20 times by eight *T. sulcifrons* on July 17, 1941. All bites occurred upon the immediate transfer of the horseflies from the infected to the susceptible horse. On July 28, eleven days following exposure to fly bites, the first reaction was noted in horse 1295. This first febrile reaction was mild. but was followed by eight intermittent reactions.

The temperature reached a maximum high of 106.6 F., and the animal showed sluggishness and muscle stiffness, but otherwise clinical

TABLE II—Passage of Wyoming Field Strain of Virus Through Horses by Injection and by the Bites of Stableflies and Horseflies

Origin	First Passage	Second Passage	Third Passage		Fourth Passage		Fifth Passage	Sixth Passage
Virus from Wyoming Outbreak	Filtered Serum Subcut.	Filtered Serum Subcut.	Filtered Serum by Different Methods of Exposure		Fly Bites and Inoculation of Mouth Parts of Infected Horseflies		Whole Blood or Filtered Serum Subcut.	By Skin Punctures with Hypodermic Needle
Horse	Horse	Horse	Amount	Horse	Amount	Horse	Horse	Horse
Acute field case	1298	1297	1 cc. subcut.	1289				
			1 cc. intradermic	1275				
			1 cc. intracranial	1252				
			1 cc. intravenous	1246	20 horsefly bites	1295		
					60 horsefly bites	1320	1267	
					Injected with mouth parts of 24 horseflies	1321	1266	1312
			1 cc. of a 1:10 dil. subcut.*	1268				
			1 cc. of a 1:1,000 dil. subcut.*	1274	233 stablefly bites	1270		

*Serum dilutions in physiologic saline.

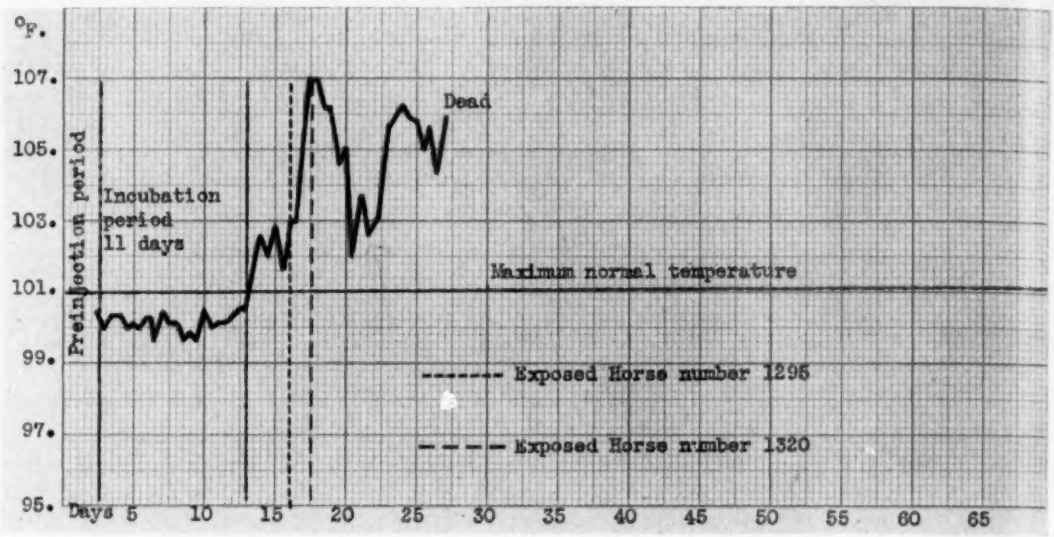
TABLE III—Comparative Data on Fly Transmissions with Other Exposure Methods and Virus Dilution

Horse No.	Virus Passage	Method of Exposure		Number of Fly Bites or Amount Injected	Kind of Material	Incubation Period (Days)	Temp. Reactions	Duration		No. Days High Temp.	Type of Disease	Termination of Disease
		Natural	Artificial					Days from Exposure Date	Days from Initial Reaction			
1298	1st		Subcutaneous injection	60 cc.	Acute Wyo. filtered serum	19	1	30	11	11	Acute	Died
1297	2nd		Subcutaneous injection	20 cc.	Acute 1298 filtered serum	12	1	24	12	12	Acute	Died
1275	3rd		Intradermic injection	1 cc.	Acute 1297 filtered serum	14	1	28	14	14	Acute	Died
1246	3rd		Intravenous injection	1 cc.	Acute 1297 filtered serum	11	1	25	14	14	Acute	Died
1252	3rd		Intracranial injection	1 cc.	Acute 1297 filtered serum	10	1	27	17	17	Acute	Died
1289	3rd		Subcutaneous injection	1 cc.	Acute 1297 filtered serum	11	1	29	18	18	Acute	Died
1268	3rd		Subcutaneous injection	0.1 cc.	Acute 1297 filtered serum	12	1	26	14	14	Acute	Died
1274	3rd		Subcutaneous injection	0.001 cc.	Acute 1297 filtered serum	25	2	51	27	26	Acute	Moribund; destroyed
1295	4th	Horsefly bites		20 bites	Acute 1246 virus	11	10	151	140	46	Subacute	Alive
1320	4th	Horsefly bites		60 bites	Acute 1246 virus	10	4	42	32	28	Acute	Died
1321	4th		Subcutaneous injection	24 horsefly mouthparts saline suspension	Acute 1246 virus	16	4	49	33	21	Acute	Died
1270	4th	Stablefly bites		233 bites	Acute 1274 virus	24	4	63	39	16	Subacute	Died
1267	5th		Subcutaneous injection	10 cc.	Acute 1320 filtered serum	8	1	31	24	22	Acute	Died
1266	5th		Subcutaneous injection	10 cc.	Acute 1320 filtered serum	10	3	37	27	20	Acute	Died
1317	5th		Subcutaneous injection	40 cc.	Subacute 1295 blood	22	3	63	41	29	Acute	Died
1312	6th		Skin punctures with hypodermic needles	21 needle punctures	Acute 1266 virus	17 to 24	1	40	16	16	Acute	Died

symptoms were not marked. The horse is still alive and the disease thus far has run a mild subacute course (graph 3). The presence of virus in this horse was confirmed by injecting 40 cc. of its whole blood (collected on October 7, or 71 days subsequent to the initial reaction) into normal horse 1317, which developed the disease in a subacute form in 22 days (table 3).

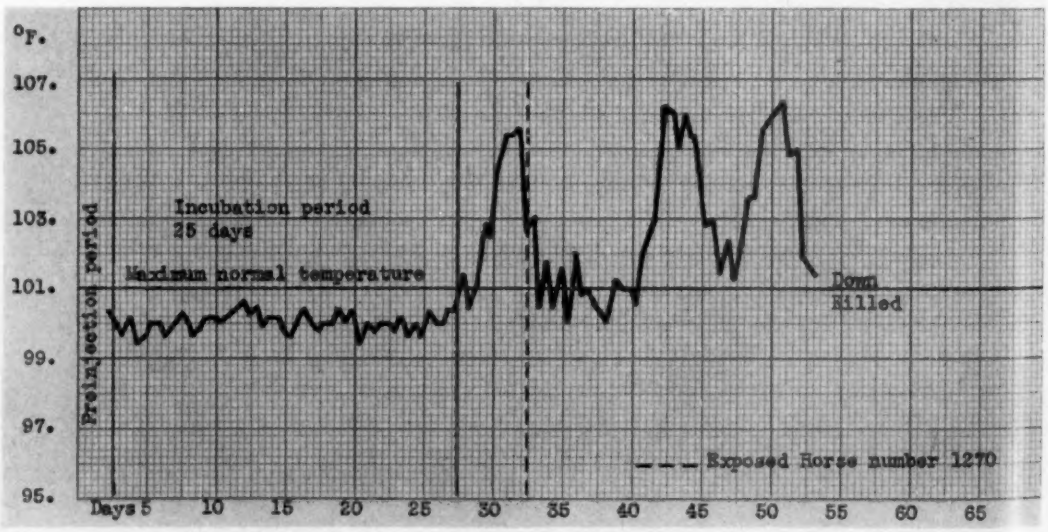
The 20-bite horsefly transmission attempt on horse 1295 was conducted on the fourteenth day following the inoculation of source animal 1246. The first symptoms in the source animal occurred July 14, or three days previous to the time the horsefly bite experiment was conducted. At the time of the experiment this source animal had a temperature of 105.4 F. and showed typical symptoms of an acute attack (graph 1).

Experiment 2.—A normal horse, No. 1320, weighing approximately 1,000 lb., was bitten 60 times by 16 *T. sulcifrons* on July 18, 1941. All bites occurred upon the immediate transfer of the horseflies from the infected to the susceptible horse. On July 28, or ten days subsequent to exposure to the horsefly bites, horse 1320



Graph 1. Temperature record on infectious anemia horse 1246 used as a source animal in horsefly transmission experiments 1, 2 and 3.

developed the usual initial febrile reaction followed by typical symptoms of acute infectious anemia, such as congestion and icterus of the conjunctiva, depression, inappetence, muscular weakness and loss of flesh. The disease ran a typical acute course, terminating in death on the fifty-second day following exposure to the fly bites (fig. 1). The initial febrile reaction reached a maximum of 106 F. and was followed by three intermittent reactions (graph 4). The necropsy and histologic examination revealed pathologic alterations characteristic of the acute form of the disease. The presence of virus in horse 1320 was confirmed by inoculating 10 cc. of its filtered blood serum into each of two normal horses (1267 and 1266). The first normal horse (1267) reacted in eight days and developed a severe acute form of the disease. The temperature reaction was more or less continuous and reached a maximum of 105.8 F. In addition to the usual symptoms a rapid loss of weight, watery diarrhea and edema



Graph 2. Temperature record on infectious anemia horse 1274 used as a source animal in stablefly transmission experiment 4.

of the sheath and legs were observed. The animal became moribund 31 days following exposure and was destroyed.

The second normal test horse, 1266, reacted in ten days, had three typical intermittent febrile reactions and died of acute infectious anemia 37 days following exposure.

A third normal horse, 1312, was in turn infected from horse 1266 by hypodermic needle puncture. This was accomplished by contaminating a sterile 20-gauge, 1½-in. hypodermic needle by first inserting it under the skin of infected horse 1266 and then under the skin of normal horse 1312. Three such punctures were made daily for a period of seven consecutive days, or a total of 21 punctures. First symptoms appeared on the sixteenth day following the date of the last three needle punctures. The disease ran an acute course and terminated in death on the sixteenth day of sickness.

This test also furnished additional evidence that mechanical transmission may be accomplished by minute quantities of infective material.

The 60-bite horsefly transmission attempt on horse 1320 was conducted on the fifteenth day following the inoculation of source animal 1246. The first symptoms in the source animal occurred July 14, or four days previous to the time the horsefly bite experiment was conducted. At the time of the experiment this source animal had a temperature of 107 F. and showed typical symptoms of an acute attack (graph 1).

Experiment 3.—On July 22, 1941, the mouthparts of the 24 horseflies used in experiments 1 and 2 (conducted July 17 and 18, respectively) were ground and 10 cc. of a saline suspension of these parts was injected subcutaneously into susceptible horse 1321, weighing approximately 950 lb. On August 7, or 16 days following the injection, symptoms appeared in this animal. The disease ran an acute course, terminating in death on the forty-ninth day after exposure. There were four intermittent febrile reactions, with a maximum high temperature of 106.4 F. (graph 5). In addition to the usual clinical symptoms associated with acute infectious anemia, rapid loss of weight and extreme muscular weakness occurred.

Experiment 4.—On August 2, 1941, by interrupted hand feeding, approximately 233 *S. calcitrans* were first allowed to feed for a short time on infected horse 1274 and then to engorge fully on susceptible horse 1270, weighing approximately 1,100 lb. Following the transmission attempts experimental horse 1270 was sprayed with fly repellent and immediately moved to another isolation stable. On August 26, or 24 days following exposure to the flies, experimental horse 1270 developed a mild febrile reaction with a maximum high temperature of 101.2 F., followed by two similar reactions at five- and ten-day intervals. Ten days following the third reaction a severe more or less continuous reaction developed which reached a maximum of 106.2 F. and persisted until death, which occurred 63 days following exposure to flies (graph 6). This horse showed typical clinical symptoms of subacute infectious anemia, including extreme muscular weakness, rapid loss of weight, marked depression and edema of the sheath and abdomen (fig. 2). Necropsy and histologic findings, while not extensive, were characteristic.

This experiment was conducted on the thirtieth day following the inoculation of the source animal (No. 1274). The first symptoms

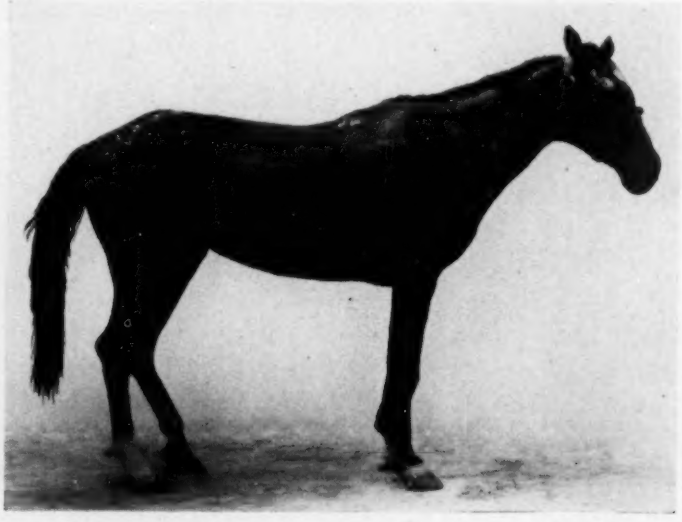
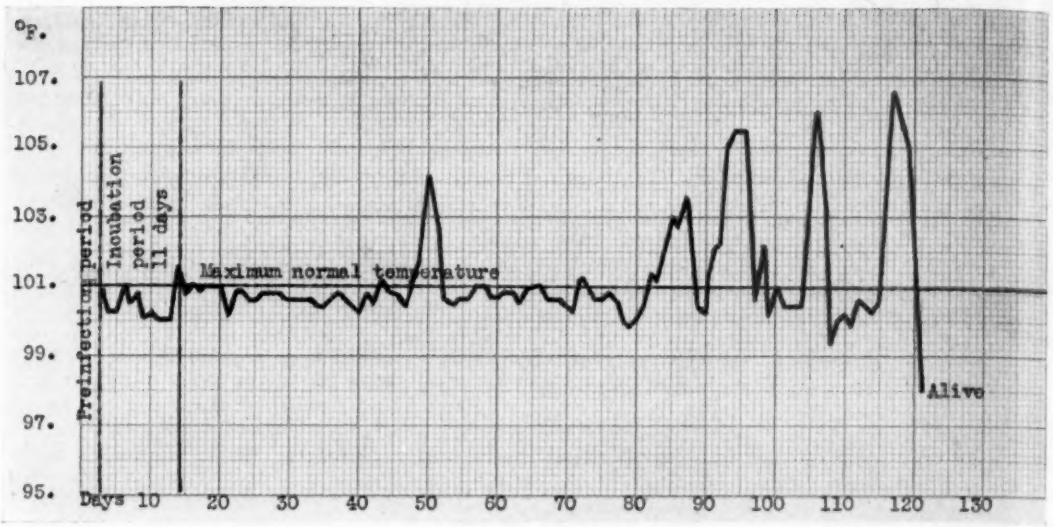


Fig. 1. Subacute case of infectious anemia, horse 1270 (two days prior to death). Infected by approximately 233 stablefly bites.

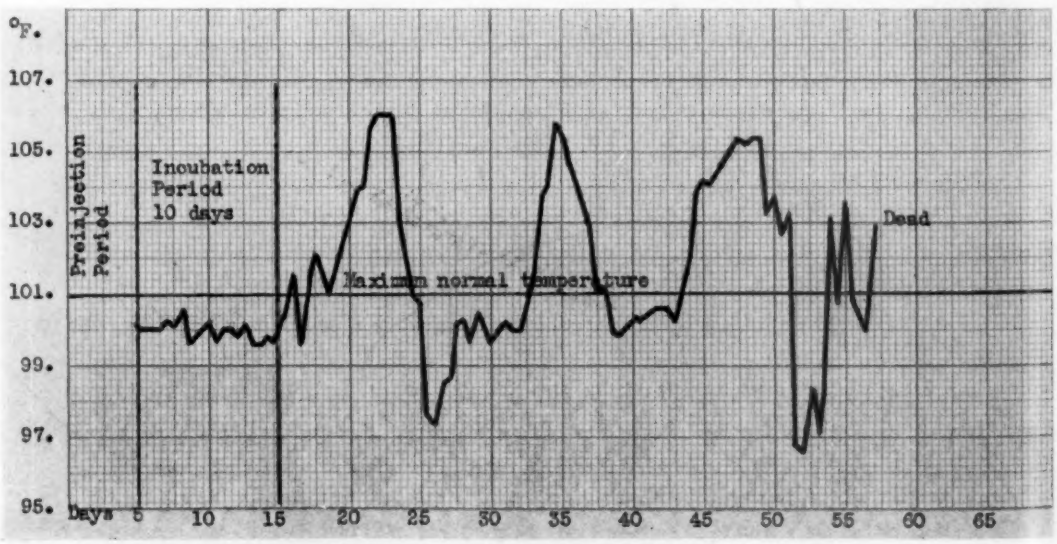


Graph 3. Temperature record on infectious anemia horse 1295 infected by 20 horsefly bites (experiment 1).

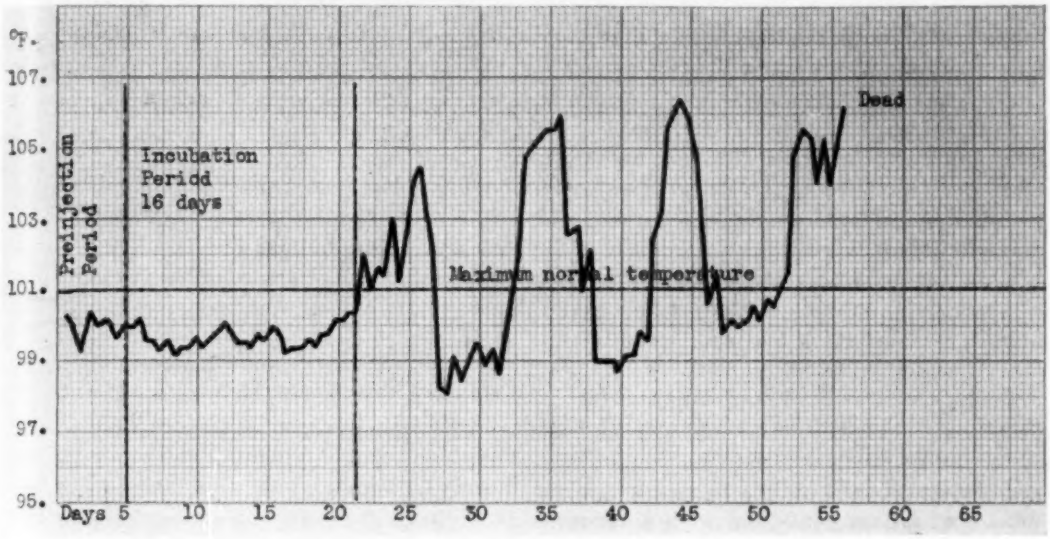
occurred in the source animal on July 28, or five days previous to the time the transmission attempt was made. At the time of the experiment the temperature of this horse was 103 F. and it showed typical symptoms of an acute attack (graph 2).

DISCUSSION

In the experiments herein described, conclusive evidence was obtained that both horseflies and stableflies may easily transmit infectious anemia from infected to susceptible horses. It also was established that the subcutaneous inoculation of the virus in 1:1,000 dilution, or 21 subcutaneous punctures with a 20-gauge, 1½-in. hypodermic needle which previously was used similarly to puncture the skin of an infected horse, was sufficient to set up the disease in the acute form in susceptible horses. This in itself tends to indicate



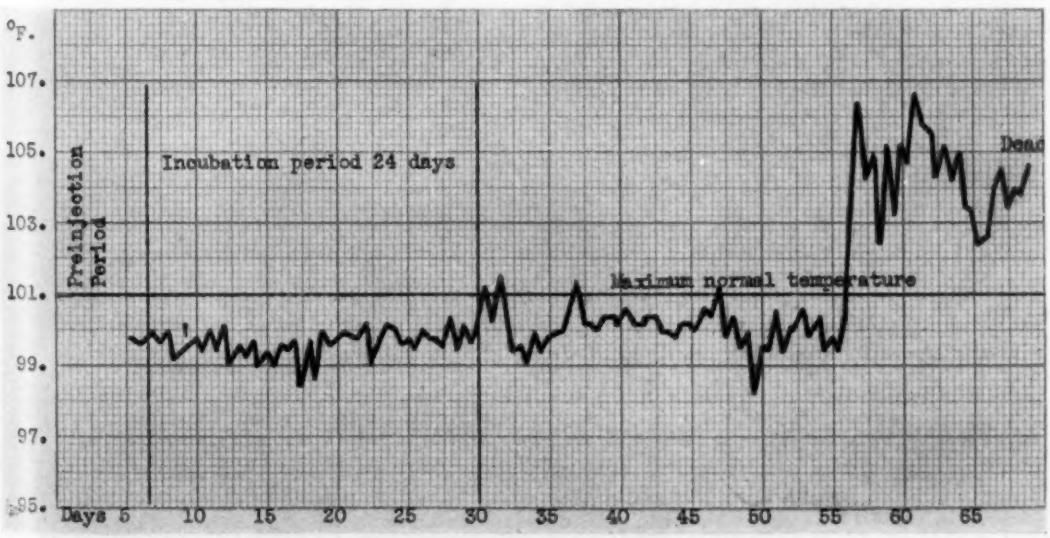
Graph 4. Temperature record on infectious anemia horse 1320 infected by 60 horsefly bites (experiment 2).



Graph 5. Temperature record on infectious anemia horse 1321 infected by subcutaneous injection of mouth parts of horseflies (experiment 3).

that the small amount of blood adhering to the mouth parts of biting flies after partially feeding on an infected animal may be sufficient to transfer the disease when feeding is completed on susceptible animals. It was further demonstrated that the mouth parts of horseflies after first feeding on a sick animal were contaminated with sufficient virus to set up the disease in a susceptible horse when inoculated subcutaneously. The probability of successful transmission by flies appears to be favored by an abundance of flies, by conditions causing interrupted feeding and by the presence in the stable or pasture of acute cases or horses carrying a virulent strain of virus.

In these experiments transfer of the dis-



Graph 6. Temperature record on infectious anemia horse 1270 infected by approximately 233 stablefly bites (experiment 4).

ease appeared to be more readily effected with horseflies than stableflies, since positive transmission with a short incubation period was accomplished in one case by 60 horsefly bites and in another case by 20 horsefly bites, whereas positive transmission with a considerably longer incubation period was effected by approximately 233 stablefly bites.

This suggests that horseflies on account of their large mouth parts carry a greater quantity of infective material and inflict a larger bite and, therefore, may play a more important rôle in transmission than stableflies.

As far as we know, this is the first investigation of this nature to be carried out with host animals infected with a virus of known origin and virulency and in which the feeding of flies on infected and susceptible animals by the interrupted method was accomplished within a few hours. It was, therefore, possible to determine the exact period of incubation in the experimentally infected animals and to compare the course and termination of this type of infection with that induced artificially by inoculation. In these experiments, the incubation period and course of the disease in fly-infected animals were similar to that induced by artificial infection.

On the basis of the experimental results obtained, it appears likely that both the horsefly and stablefly are important factors in the dissemination of infectious anemia. It is evident, therefore, that fly control in areas where the disease is prevalent constitutes one of the most important safeguards against dissemination of the infection.

Since these studies have also demonstrated that transmission can be effected by injections of minute quantities of virus, certain other factors are important in the control of the disease. The greatest care always should be used to prevent transmission of the disease from animal to animal by the use of unsterilized instruments, bleeding needles or hypodermic needles.

The common use of equipment that may produce skin abrasions, such as bridles, harness, brushes and curry combs, on both infected and healthy horses is probably dangerous and should be avoided.

SUMMARY

Transmission of infectious anemia by arthropods is reviewed. Data are reported to indicate that infection in a susceptible horse can be induced by the subcutaneous inoculation of 1 cc. of virus in 1:1,000 dilution, or by repeated pricking of the skin with a hypodermic needle contaminated with virus. The researches of Scott,[8] the Japanese Commission,[1] Howard[10] and Flocken of the Bureau of Animal Industry are further clarified.

The evidence reported on fly transmission of infectious anemia in this paper confirms the work of Scott[8] and supports the

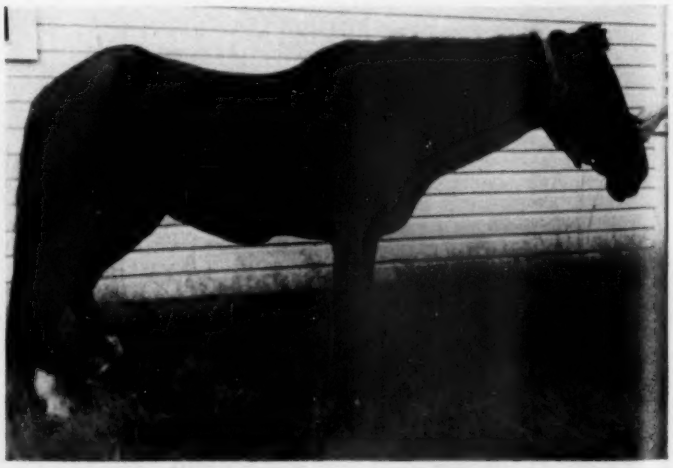


Fig. 2. Acute case of infectious anemia, horse 1320 (four days prior to death). Infected by 60 horsefly bites.

contention that biting flies probably play an important rôle in the dissemination of infectious anemia. Three positive cases of fly transmission are reported. Two susceptible horses developed infectious anemia ten to eleven days after being bitten by the horsefly, *Tabanus sulcifrons*, and one susceptible horse 24 days after being bitten by the stablefly, *Stomoxys calcitrans*.

Conclusive evidence also was obtained that the mouth parts of horseflies subsequent to feeding on an infected horse contained virulent virus.

ACKNOWLEDGMENT

We are indebted to Dr. R. L. Knudson, Pathological Division, Animal Disease Station, Beltsville Research Center, Beltsville, Md., for assistance rendered in the horsefly exposure experiments.

Notes on Susceptibility of the Golden Hamster (Cricetus Auratus) to Equine Encephalomyelitis Virus*

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THE PROLIFICACY, small size and possible adaptability of hamsters to various biologic studies are being more and more widely considered. These rodents have been utilized in connection with investigations of tuberculosis,[1-3] leishmaniasis,[4] rabies,[5] sarcoma,[6] leprosy,[7] brucellosis[8] and other diseases. Recently hamsters have been experimentally infected with St. Louis encephalitis virus[9] and American equine encephalomyelitis virus.[10] The experiments here recorded confirm the report by Mitchell and Walker,[10] with some added details.

Two strains of virus, one eastern type and one western type, recovered from typical clinical field cases of equine encephalomyelitis were used in the inoculation tests. Both strains of virus (the western from a North Dakota horse, No. 1415-W, and the eastern from a Maryland horse, No. 1418-E) were "isolated" in the Bureau during August 1941. Both had been propagated in series by intracerebral inoculation of guinea pigs and prior to the pathogenicity tests here noted were found to be filtrable and type specific.[11]

Young, virgin female hamsters were injected while under ether anesthesia, with culturally sterile saline suspension of brain containing virus of the third passage, or generation, in guinea pigs (table I).

All injected animals were active and apparently normal on the day following injection. On the subsequent days noted in the table, symptoms such as depression, incoördination and ruffled coat appeared. These were intensified and followed by somnolence, slow respiration and prostration. Until apparently completely paralyzed, a number of the animals continued to pack feed into the pouches. The brains of the animals dying of the disease, or killed by chloroform inhalation when showing definite symptoms, were removed aseptically and found to be culturally sterile. One half of the brain of each hamster was immediately placed in 15 per cent formalin for histopathologic study and the other retained separately for preparation of a suspension for intracerebral inoculation of guinea pigs to demonstrate the presence of virus. When harvesting the hamster brains, the left hemisphere of one was reserved for microscopic examination and the right used for virus passage. In the animal remaining from each pair injected with one virus, the opposite allotment was made, so that of the hemispheres into which virus was introduced in the two animals given eastern or western virus, one from each animal was from the injected side, the other from the opposite side.

Sufficient virus was present in all preparations to induce typical encephalomyelitis in two guinea pigs used as checks. A hamster and a guinea pig injected with a suspension of brain tissue from a normal guinea pig developed no abnormality and were chloroformed to death on the fifth day

*From the Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture.

TABLE I

No. Injected	Virus	Route	Dose	Dilution	Results
2	1418:E	I.C.	0.1 cc.	1:100	Symptoms 2nd day; 1 died and 1 killed 3rd day
2	1418:E	S.C.	0.5 cc.	1:10	Symptoms 3rd day; 1 died and 1 killed 4th day
2	1415:W	I.C.	0.1 cc.	1:100	Symptoms 3rd day; 1 killed 3rd day, 1 killed 4th day
2	1415:W	S.C.	0.5 cc.	1:10	Symptoms 4th day; 2 dead 6th day

Contemporary titrations of each virus were conducted in guinea pigs (two guinea pigs for each dilution), the dose being 0.1 cc. I.C. (intracerebrally). The dilutions used and the results are summarized as follows, the numerator of each fraction following the dilution being the number of guinea pigs developing encephalomyelitis and the denominator being the number injected:

Eastern virus (1418-E)—1:500, 2/2; 1:1,000, 2/2; 1:10,000, 2/2; 1:50,000, ½; 1:100,000, ½; 1:300,000, ½; 1:500,000, 0/2.

Western virus (1415-W)—1:500, 2/2; 1:1,000, 2/2; 1:10,000, 2/2; 1:50,000, 0/2; 1:100,000, ½; 1:300,000, 0/2; 1:500,000, 0/2.

From the titration in guinea pigs it may be concluded that the hamsters injected intracerebrally with eastern virus (1:100) received at least 500 guinea pig m.l.d. and those injected subcutaneously (S.C.) with 1:10 dilution of virus received at least 25,000 guinea pig m.l.d. It follows that the hamsters injected intracerebrally with western virus received 100 guinea pig m.l.d. and those injected subcutaneously received 5,000 guinea pig m.l.d.

after injection as pathologic controls, together with a normal uninjected hamster.

The histopathology in the brains of the hamsters injected with virus varied somewhat in the individual cases. Congestion and hemorrhages in the meninges and perivascular hemorrhages and small blood extravasations in the brain substance were more or less constant. Cell infiltrations of varying density were observed in the pia-arachnoid and diffusely in the white matter and cortex of the cerebrum and other portions of the brain. The cells were polymorphonuclear, mononuclear and lymphoid, with occasional large macrophages. Perivascular cuffing, varying from the presence of a few cells to considerable aggregations in some cases, was found. Karyorrhexis was observed in the cases with more marked lesions. Vacuolation of the intercellular ground substance was present in most of the brains and definite foci of necrosis were observed in some. No differences of a specific character were found in the pathology due to the two types of virus. In general, the pathology resembled that in the guinea pig, as described by King.[12]

CONCLUSIONS

The golden hamster (*Cricetus auratus*) is susceptible to both western and eastern equine encephalomyelitis virus injected intracerebrally or subcutaneously.

The pathology of the disease in hamsters corresponds to that found in guinea pigs.

References

[1]Korns, J. H., and Lu, G. Y. C.: The diagnosis of tuberculosis by means of the hamster. Am. Rev. Tuberc., xvii (1928), pp. 279-281.

[2]Lu, G. Y. C.: Further study on susceptibility of the hamster (Chinese field mouse) to the tubercle bacillus. Am. Rev. Tuberc., xx (1929), pp. 938-942.

[3]Griffith, A. S., and Pagel, W.: Susceptibility of the golden hamster (*Cricetus auratus*) to bovine, human, and avian tubercle bacilli and to the vole strain of acid-fast bacillus (Wells) with histological observations. J. Hyg., xxxix (1939), pp. 154-160.

[4]Mathervassjan, S. T., and Zaturjan, A. T.: Experimentelle infektion des armenischen hamsters Cricetulus migratorious mit Leishmania donovani. Arch. f. Schiffs-u. Tropenhyg., iii (1933), pp. 190-193.

[5]Yen, A. C. H.: Experimental virus infections in Chinese hamster. I. Susceptibility to fixed rabies virus. II. Susceptibility to street rabies virus. Proc. Soc. Exp. Biol. & Med., xxxiv (1936), pp. 315-318, 648-651.

[6]Gye, W. E., and Foulds, L.: Note on the production of sarcomata in hamsters by 3:4—benzpyrene. Am. J. Cancer, xxxv (1939), p. 108.

[7]Transmission of human leprosy to the hamster. Lancet, ccxxxv (1938), p. 1306.

[8]Tchernomoretz, I., and Ellenbogen, V.: Note on susceptibility of the Syrian hamster (*Cricetus auratus*) to *Br. abortus*. J. Comp. Path. & Therap., l (1937), pp. 136-140.

[9]Broun, G. O., Muether, R. O., Mezera, R. A., and Le Gier, M.: Transmission of St. Louis encephalitis to the hamster. Proc. Soc. Exp. Biol. & Med., xlvi (1941), pp. 601,603.

[10]Mitchell, C. A., and Walker, R. V. L.: Studies in equine encephalomyelitis susceptibility of some mammals and birds. Canadian J. Comp. Med., v (1941), pp. 314-319.

[11]Shahan, M. S., Giltner, L. T., and Osteen, O. L.: The isolation and typing of equine encephalomyelitis virus. Cornell Vet., xxx (1940), pp. 151-160.

[12]King, L. S.: Studies on eastern equine encephalomyelitis. I. Histopathology of the nervous system in the guinea pig. J. Exp. Med., lxviii (1938), pp. 677-692.

Observations on a Fungus Skin Disease of Iowa Muskrats*

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IN CONNECTION WITH ecologic studies of muskrats *(Ondatra z. zibethicus)* begun in 1934, approximately 3,500 animals of newborn to subadult sizes were examined in central and northwestern Iowa. Nearly 700 of these were marked when very young, chiefly by means of serially numbered metal tags.[1, 2] Members of about 400 litters were studied while still under care of their parents in natural habitations; many were repeatedly weighed and measured, some as often as 16 times at daily intervals.

Individuals less than a month old, and some older ones, were caught by hand in or near the chambers of marsh lodges and were generally released promptly and in a manner calculated to be least upsetting to their living routine. Tagged animals in particular were examined as opportunities permitted, though after their first month specimens soon become so wary and active that they could no longer be taken uninjured and had to be shot. Most of the tagged subadults yielding valuable data were, upon my request, trapped by the Conservation Commission in early winter from state-owned marshes on which intensive summer investigations had been made.

It was observed during the 1935 studies at Round Lake, northwest of Ruthven, that considerable numbers of suckling young were afflicted by what looked like a fungus disease of the skin. On July 4, an assistant waded about muskrat lodges to help capture swimming young for tagging, and some days thereafter became aware of "ringworm" on her legs—an infection later attributed to a diseased litter in the vicinity of which we had worked for perhaps an hour. The following summer I contracted on an arm what was presumably a like ailment and submitted cultures to Miss Vera K. Charles of the Mycology and Disease Survey, Bureau of Plant Industry, U. S. Department of Agriculture. After various failures I managed, in 1938, to obtain for Miss Charles some infective material from young muskrats. On the basis of comparative cultural studies[3] she concluded that *Trichophyton mentagrophytes* (Robin) Blanchard was the causative organism both of my arm infection and of the disease commonly occurring on the muskrats.

MANIFESTATIONS AND COURSE OF THE DISEASE IN MUSKRATS

My field notes give many symptoms for the condition referred to as "skin disease," but the appearance of disease concurrently among members of certain litters, with exhibitions of the whole range of symptoms, indicates but one major infection. Moreover, Charles[3] writes:

> It is believed that only one organism was involved in the skin disease of muskrats because only one organism was consistently found in the material submitted for examination, and in the cultures isolated from the skin and hair of diseased animals. All the other fungi that appeared in culture or were present on the fresh material were clearly secondary. A species of Fusarium was observed in one culture, but it is not thought that a species of this genus would produce the clinical symptoms presented in this case.

Of course, in so far as young muskrats are found sick or dead from other diseases or from unrecognized causes and as only a limited proportion of diseased yet living specimens could be taken without interfering with the primarily ecologic objectives

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[1]Errington, P. L.: Observations on young muskrats in Iowa. J. Mamm., xx (1939), p. 465.

[2]Errington, P. L., and Errington, C. S.: Experimental tagging of young muskrats for purposes of study. J. Wildlife Mgt., i (1937), p. 49.

[3]Charles, V. K.: A ringworm disease of muskrats transferable to man. J. Washington Acad. Sci., xxx (1940), p. 338.

of the research, we can not rule out the possibility that unknown infections, physiologic deficiencies, etc., may have contributed to the stunting and mortality ascribed to the fungus.*

Conspicuous watery or suppurated pustules—frequently in ring formation on underparts in contact with wet nests—were seen on many diseased, week-old young. Swellings about the ears and anal region were sometimes recorded, as were incrustations and scurf. Victims commonly stopped growing, then lost weight and died as their infections became severe. The ventral surfaces of badly afflicted individuals in their late short-hair pelage, or at the age of about two weeks,[1] took on a transversely striped, corrugated appearance from skin folds.

Dandruff-like scurf and bald spots on top of the head, back and similar places that were normally kept more or less dry were mostly seen on animals having the soft, woolly pelage of their third and fourth weeks.[1] Although blisters on the naturally rather bare tail and feet were not unusual, the disease was manifested on these older animals not so much by pustules as by a patchy hairlessness of extremities, face and throat, ventral thorax and abdomen, and by poor growth and death. Often the recognizably affected skin areas on some members of unquestionably diseased litters would be surprisingly small or even absent, and the principal evidence of disease would be the unthriftiness and mortality common to the greater part, or the whole, of the litter.

Fungus infections recognized on animals less than 2 weeks old were lethal without recorded exception, and all animals known to have recovered had reached or passed their fourth week without visible signs of the disease other than a few blisters at the tip of the tail—which tip they generally chewed off. Of 85 infected individuals listed in my Round Lake notes, 1935-38, seven in their fifth and sixth weeks appeared hopelessly diseased. Fair circumstantial evidence (unsupported by tagging data) suggests that a partly hairless animal, having an estimated age of between 40 and 50 days when first seen, September 4, 1936, was alive but undersized by October 9. No evidence of active skin disease was seen on about 1,600 adults and subadults of fall and winter (including 796 from Round Lake). Two tagged animals collected in winter as subadults were recognized survivors of light, late infections; both had trimmed their tail tips months previously but otherwise were full sized and in excellent condition.

The sequences of the disease were traced for an average of 14 days in 13 litters having five or more young in each. Table I illustrates comparative growth of diseased and "normal" members of these litters.

Poor growth rates usually could be noted several days before the appearance of skin lesions or bald spots, and susceptibility in many cases was probably conditioned by lack of vigor from other causes, especially in the very young. Variations in sizes of healthy muskrats may be pronounced at all ages, and differences may be seen among members of given litters from birth,[1] but the likelihood of congenital "runts" ultimately showing the first pustule in diseased litters is too great to be coincidental. Nevertheless, few other members escaped infection if its salient manifestations appeared in the litter within twelve days after birth.

INCIDENCE AND SEVERITY OF INFECTION

Thirty-five, or 9.6 per cent, of 364 northwest Iowa litters (305 from Round Lake and 59 from neighboring marshes) worked with during the breeding seasons of 1935, 1936 and 1938 contained skin-diseased young. The relation of time of year to incidence and severity of infection is shown in table II.

Only 4, or 2.8 per cent, of 141 of the litters born in April and May were infected; and, of the 18 known members of infected litters, 8, or 44.4 per cent, caught the disease, and 6, or 75 per cent, of the 8 diseased animals evidently died. Of 208 litters

*Acknowledgments for coöperation in handling miscellaneous diseased specimens of muskrats are due to, among others, Drs. E. A. Benbrook and E. R. Becker (Iowa State College), B. V. Travis (formerly of Iowa State College, now of the U. S. Bureau of Entomology), R. G. Green (University of Minnesota), and J. E. Shillinger (U. S. Fish and Wildlife Service).

born in June and July, 27, or 13 per cent, were infected. The figures arrived at for infected animals in the diseased litters and for infected animals dying were 78, or 75 per cent, of 104 and 72, or 92.3 per cent, of 78, respectively. The data for August show 4, or 26.7 per cent, of 15 litters infected and a complete loss of the infected litters—12 of 12 known members both infected and dying.

Not only did the early litters have a much lower incidence of infection, but the consequences of litters becoming infected also tended to be less serious, principally because of the larger size the animals attained before the disease appeared. Seldom would fewer than half of the members of a litter succumb if unmistakable evidence of infection could be seen in the litter by the end of the third week after birth. Animals still healthy when weaned " . . . in most cases early in the fourth week"[1] seemed practically safe from lethal attack by the fungus. Charles[3] was unable to obtain a culture from the dry skin of a subadult of 185 days (No. 2732 of table I) that had recovered from a tail infection.

Inasmuch as all cultures of Trichophyton isolated by Charles from muskrat specimens were from victims losing hair, hair-slipping stages would seem particularly important in direct transmission. From a study of the circumstances preceding and attending manifestations of the contagion in litters, however, I am inclined to feel that the spores may be mainly water-borne. Helpless young are frequently carried submerged by their mothers from lodge to lodge,[1, 4] are splashed by, and come in contact with, the wet bodies of active animals, especially their parents, or they may become soaked through partial submergence of the nests in which they are kept.

The question as to what degree handling of the young may have aided the spread of skin disease from litter to litter was given consideration. Twenty-two, or 62.9 per cent, of the 35 litters affected showed symptoms at first visits to lodges or under other circumstances eliminating any possibility of their having been infected by the investigators; for 7, or 20 per cent, it was possible but improbable that infection may have been man-transmitted; for 6 (5 in 1936, 1 in 1938), or 17.1 per cent, a fair but by no means convincing possibility of infection from handling was recognized.

Only 5 litters of young were known to have been kept in semi-permanent lodges that housed infected litters earlier in the season; 1 of these subsequent litters became infected; 2 apparently did not, at least up to the age of weaning; and the fate of the other 2 was not determined.

The earliest cases of the disease on Round Lake were distributed much at random each year, though some localization was noted in midsummer. In 1935, five of 12 lodges harboring infected litters were in one corner of the marsh, 3 were in another corner, 2 in another; in 1936, four of 13 were clumped, and 6 others occurred somewhat in pairs; in 1938, two of 9 were close together, and another had 2 consecutively infected litters.

In late summer, when the proportion of diseased to healthy suckling young appears to rise, most of the litter-containing habitations are small, temporary and constructed of fresh aquatic vegetation. The nests may be but a few inches over the water, or even in it, and thus the young lose what little sanitary advantage fresher quarters would seem to afford. For that matter, the young which are kept in mouldering old lodges seem no more subject to infection than litters elsewhere.

Shillinger[5] has written upon the relation of low water to massive infestations of coccidia in muskrats and his findings doubtless pertain to other diseases. Many are the significant variables that drouth introduces into the ecology of the muskrat,[6] but, as far as we can see, the incidence of litters infected with skin disease seemed independent of water levels on Round Lake (table III). Although quantitative data on the Round Lake litters were not obtained in 1937 and 1939, a basis for comparison is afforded by the percentage of winter-caught

[4]Errington, P. L.: Drowning as a cause of mortality in muskrats. J. Mamm., xviii (1937), p. 497.

[5]Shillinger, J. E.: Coccidiosis in muskrats influenced by water levels. J. Wildlife Mgt., ii (1938), p. 233.

[6]Errington, P. L.: Reactions of muskrat populations to drought. Ecology, xx (1939), p. 168.

TABLE I—Weights and Measurements of Recognizably Diseased and "Normal" Litter Mates in Medium-Sized to Large Litters, Presented for Intervals Sufficient to Illustrate Trends*

Field Number of Lodge Group	Date of Observation	Age of Young Animals	Tag Number or Other (If Any) Designation of Individuals Yielding Data, Below Which, in Each Column, the First Figure Refers to Weight in Grams; the Second, to Total Length Measurements in Millimeters								Remarks
1935 No. NE23			2399	2396	2397	2398	2400				No. 2399 was probably an early victim; 2400, by July 9, was acting as if infected
	June 26	7 days	All young approximately 40–140								
	July 7	18 days		*67–191*	*63–178*	*60–178*	89–210				
	July 9	20 days			*66–178*	*66–178*	83–204				
1935 No. NE16			2467	2470	2610	2468	2469	2611	2612		Litter was slightly undernourished and backward in growth until weaning
	July 7	15 days	75–187	70–192	74–191	83–197	79–204	75–197	80–197		
	July 13	21 days	*88–221*	*74–197*	85–211	109–229	97–229	89–221	93–212		
	July 16	24 days			*91–216*	128–236	115–242	102–236	110–229		
	July 19	27 days			*90–229*	136–254	128–261	119–236	122–242		
1935 No. NE2			AC	AB	Male	AE	Female	2634	2631		
	July 6	3 days	19–90	26–121		22–108	26–115	26–121	26–115		
	July 11	8 days	*22–109*	*35–135*	*35–135*	38–134		52–150	54–142		
	July 14	11 days				*46–140*		66–172	66–165		
1935 No. 36			Female	Male	Male	Female	Female	Male			Animals listed left to right in order of severity of infection
	July 24	10 days	*40–146*	*37–140*	*43–154*	*42–154*	*50–163*	55–163			
1936 No. 59				CL3	2714	2715	2716				Diseased animals were showing pronounced hairlessness on June 20 and it is suspected that No. 2714 had already died and dispeared
	May 25	3 days	Eight young averaging 28-113, with extremes of 25-112 and 31-115								
	June 13	22 days		105–245	106–259	115–248	115–254				
	June 20	29 days	*111–235*	*118–242*			148–274				
1936 No. 35			2692	BR		2689	2713	2690	2691	B0-1	By June 19, No. 2692 was dead, and 2689, 2690 and B0-1 were probably so active that they escaped handling
	June 7	15 days	*64–193*	*62–193*		73–212	72–197	76–204	77–204	75–210	
	June 12	20 days		*85–197*	97–237	97–244	97–244	105–245	105–272	107–248	
	June 19	27 days	*80–242*		95–261		121–262		163–287		
1936 No. 107			2732	2731	BA	Female	Female				The infected young had blistered and chewed tail tips by July 5; when collected at the age of 185 days, No. 2732 was in excellent condition. The slower growth of BA was due to an injury
	June 17	8 days	45–140	48–146	38–127	43–140	43–138				
	June 23	14 days	85–185	95–200	65–170						
	June 28	19 days	120–229	155–260	97–221						
	July 5	26 days	*140–260*	*164–296*							
1936 No. 120					2742	2743	2744	2735	2745		Animals not handled on July 5 were too active to catch and probably escaped serious infection
	June 28	19 days	91–229	98–229	98–229	96–233	97–229	93–216	100–235	103–242	
	July 5	26 days	*79–210*	*105–229*	*103–224*			130–254			
1936 No. 93			2751	Female	2752	2753	2754	FF			
	June 20	7 days	38–145	37–140	42–145	44–144	45–146	43–146			
	June 25	12 days	59–172	55–170	65–178	69–178	70–178	69–175			
	June 30	17 days	*73–204*	73–211	80–220	83–214	86–226				
1936 No. 68			Female	Male	Male	Female	Male	Male	Male		
	June 21	7 days	*28–123*	*28–130*	*30–130*	36–135	40–144	42–138	45–148		
1936 No. 112			DX	DW							Both young were somewhat undersized for their age at 13 days, though only one was unquestionably diseased
	June 27	4 days	Six young averaging 28-116, with extremes of 26-112 and 29-119								
	July 6	13 days	*60–185*	57–178							
1938 No. 59	June 23	6 days	Five young were about 135 mm. in length								Individual weight and measurement data not taken
	July 3	16 days	Five diseased and stunted young about 165 mm. in length; most "normal" young of this age and litter size exceed 200 mm. in length								
1938 No. 183	June 17	7 days	Seven of nine young were healthy in appearance and about 140 mm. in length; of the other two, one was about the same length although in poor condition, and one of 127 mm. was dying of skin disease. A culture showed an Alternaria, considered non-pathogenic								Individual weight and measurement data not taken except for No. 2842 at the time it was collected. The one dying on June 17 was taken for a specimen, and four others of the litter were drowned between June 17 and 19
	June 19	9 days	Four young surviving drowning and disease were tagged (2842-45); of these, No. 2844 was undersized and had scurf and pustules about anus from which a species of Fusarium was isolated, but which was thought to have represented a contamination								
	July 16	36 days	No. 2842 collected. Its weight of 215 Gm. and length of 305 mm. did not indicate stunting, but it had denuded areas on feet and base of tail. This was a typical infection yielding Trichophyton								

*Weight and measurement data on recognizably diseased animals are italicized.

young of the year having healed stub tails of the kind resulting from chewing off diseased tips during preceding months. Of the young taken by the Conservation Commission following summer studies, 5, or 3.8 per cent, of 132 had in 1936 the type of stub tails we were watching for; 2, or 1.2 per cent, of 164, 1937; 2, or 1.2 per cent, of 167, 1938; 4, or 2.4 per cent, of 168, 1939. These criteria, whatever may be their deficiencies, do not indicate that the proportions of young that recovered from the disease in 1937 and 1939 differed very greatly from the proportions in the 1936 and 1938 seasons of intensive study; hence, it would seem probable that the incidences of infected litters in 1937 and 1939 also may have been about the same as for 1936 and 1938, or in the vicinity of 12 per cent.

Neither do the available data (table III) reveal a direct correlation between incidence of infection and population density of muskrats on the marsh as a whole; some of the indirect relationships between such incidence and density will be discussed in the next section.

EFFECT ON POPULATION

Charles[3] writes: "As far as our information goes this is the first report of *T. mentagrophytes* on muskrats." The U. S. Fish and Wildlife Service has information on some possible cases of the disease in Maryland, but, aside from this, I have been unable to learn either from literature or correspondence whether symptoms have been observed in muskrats outside of Iowa.

Within the state, by far the greatest number of cases were recorded from the Ruthven area, which is entirely to be expected for the reason that most of the work with young animals was done there. In the summer of 1939, a diseased young was encountered at Cheever Lake, which is about 15 miles from Round Lake. The studies did not involve systematic handling of litters, but 2, or 2.6 per cent, of 77 subadults trapped by the Conservation Commission in January 1940 had tails that were apparently disease-stubbed. Three, or 0.7 per cent, of 456 subadults taken by private trappers from four streams in Story county, central Iowa, 1937-40, had the same kind of stub tails, and a virtually unmistakable case of hair-slipping skin disease was seen in this county in 1936. The disease was not observed among 31 litters examined, 1937-40, on Wall and Little Wall lakes, which are situated in Wright and Hamilton counties, between Ames and Clarion, but 1, or 1.8 per cent, of 56 Wall Lake young of the year examined in November

TABLE II—Seasonal Distribution of Muskrat Litters and Incidence and Severity of Skin Disease, Northwest Iowa, 1935, 1936 and 1938*

PERIOD OF BIRTH	NUMBER OF LITTERS HAVING DISEASED MEMBERS	NUMBER OF YOUNG DISEASED† AMONG MEMBERS OF INFECTED LITTERS	NUMBER OF DISEASED INDIVIDUALS DYING‡
April 1 to 15	0 of 0		
April 16 to 30	0 of 24		
May 1 to 15	1 (1.8%) of 56	1 (25.0%) of 4	0 of 1
May 16 to 31	3 (4.9%) of 61	7 (50.0%) of 14	6 (85.7%) of 7
June 1 to 15	11 (15.3%) of 72	33 (76.7%) of 43	29 (87.9%) of 33
June 16 to 30	8 (11.3%) of 71	24 (75.0%) of 32	23 (95.8%) of 24
July 1 to 15	6 (13.0%) of 46	18 (69.2%) of 26	17 (94.4%) of 18
July 16 to 31	2 (10.5%) of 19	3 (100.0%) of 3	3 (100.0%) of 3
August 1 to 15	3 (30.0%) of 10	7 (100.0%) of 7	7 (100.0%) of 7
August 16 to 31	1 (20.0%) of 5	5 (100.0%) of 5	5 (100.0%) of 5
September 1 to 15	0 in 0		
Totals	35 (9.6%) of 364	98 (73.1%) of 134	90 (91.8%) of 98

*Figures include previously published data on seasonal distribution of litters, 1935 and 1936 (Errington, P. L.: The breeding season of the muskrat in northwest Iowa. J. Mamm., xviii, 1937, p. 333).
†Total numbers include 85 known and almost certain, and 13 probable, victims.
‡Total numbers include 77 known and almost certain, and 13 probable, fatalities.

1940 had a stub tail that was not quite typical of the disease but may have been due to that cause.

Dodge[7] cites literature showing the horse to be a host for varieties of *T. mentagrophytes*. The possibility that farm animals, with their various imperfectly recognized "ringworm" diseases, are a factor in the distribution of *T. mentagrophytes* spores in muskrat waters has not been explored.

A disease that may prove to be fairly widespread and one killing the majority of the members of 9.6 per cent of the 364 muskrat litters worked with on the principal study area can hardly be ignored in an analysis of population recovery, but the net effect of the disease on the increase and maintenance of muskrat populations is less than might at first glance be surmised.

The net effect of the disease mortality on recovery of muskrat populations was, for one thing, lessened by adjustments on the part of the muskrats themselves. It has become apparent that poor success in raising young early in the summer may stimulate adults to prolong their breeding and to give birth to extra litters—the third or fourth for the season for individual females—in July or August. Of the 35 diseased litters referred to in table II, the losses in 13, while heavy, were judged to have been offset by later breeding. Much of the breeding, in all likelihood, would not have taken place except in response to the preceding failures. Twenty-two of the diseased litters represented, as far as could be ascertained, the final offspring for the season for their respective mothers, and mortality in these was calculated to have accounted for 55, or 68 per cent, of 81 known members; 14 of the 35 diseased litters, or 3.8 per cent, of the 364 litters handled, were a virtual loss, with slight chance of compensation through further reproduction.

Other population mechanisms also may mitigate the disease losses. Although our investigations of breeding and mortality of muskrats are as yet insufficiently complete for publication in detail, the seven years of data in hand indicate that the numerical status of muskrats is to a considerable extent self-limiting, at least within the restrictions imposed by climate and habitat[6] and by human overexploitation.[8] The number of young reared on a given area under comparable conditions tends to vary inversely with the density of the breeding stock (see, for example, the data for the high-water years in table III), and accelerated mortality usually reflected either crisis or crowding. Losses of the immature from diseases, predatory enemies, intraspecific strife and miscellaneous causes were intercompensatory enough to act collectively, some automatically declining as others rose in importance as lethal agencies.

Turning again to table III, we may see that, in 1935, the incidence of skin disease in the litters on Round Lake was the lowest recorded, but that the net productivity by late fall of young per adult female was the lowest for any high-water year. The severity of losses from other causes may be regarded as a consequence of overpopulation, and these other loss rates in turn showed intercompensatory trends; on parts of the marsh where losses from intraspecific strife were heavy, predatory losses were light, and *vice versa*.

The drouth year of 1936 was one of low net productivity and of increased losses from skin disease and miscellaneous causes, though intraspecific strife had moderated and predatory losses, despite response of minks *(Mustela vison)* to vulnerability of local drouth-exposed groups,[6] had decreased with relief from the crowding of the year before. In 1937, a comfortably situated breeding population had a season that may be considered productive for Round Lake; the negligibility of losses of young from predation was evidently offset by disease, intraspecific strife and miscellaneous mortality. Regardless of the lower population of breeding muskrats, the 1938 incidence of skin disease was slightly the highest noted, yet, with continued insignificance of predation and the diminishing rôle of intraspecific and miscellaneous mortality, the season's net productivity per adult female was the highest. The poor productivity for

[7]Dodge, C. W.: Medical mycology; fungus diseases of man and other mammals (C. V. Mosby, St. Louis, 1935).

[8]Errington, P. L.: Natural restocking of muskrat-vacant habitats. J. Wildlife Mgt., iv (1940), p. 173.

TABLE III—Skin Disease and Other Mortality Factors in Relation to Recovery of Muskrat Populations During Five Breeding Seasons, 1935-39, Round Lake, Clay County, Iowa

SEASON	WATER LEVELS	AVERAGE BREEDING DENSITIES OF ADULT FEMALES	AVERAGE LATE FALL COMBINED DENSITIES OF ADULT AND YOUNG	AVERAGE NUMBER OF YOUNG RAISED PER ADULT FEMALE	PERCENTAGE OF LITTERS INFECTED WITH SKIN DISEASE*	APPRAISAL OF LOSSES OF YOUNG FROM PREDATION	APPRAISAL OF LOSSES OF YOUNG FROM ATTACK BY OTHER MUSKRATS	APPRAISAL OF LOSSES OF YOUNG FROM MISCELLANEOUS CAUSES
1935	High†	0.58 per A.	4.44 per A.	5.6	6.9%	Severe	Severe	Severe
1936	Low, summer and fall‡	0.18 per A.	1.00 per A.	4.4	12.0%	Light	Moderate	Severe
1937	High	0.19 per A.	1.53 per A.	6.8	About 12.0%	Negligible	Probably moderate	Probably moderate
1938	High	0.14 per A.	1.41 per A.	8.3	12.2%	Negligible	Fairly light	Fairly light
1939	Low in fall	0.44 per A.	2.84 per A.	5.4	About 12.0%	Negligible	Probably severe	Severe

*For source of 1937 and 1939 figures, see comparison of stub-tailed specimens of subadults and discussion in text.
†During no seasons within the period of study were water levels sufficiently high to change the marsh into an open water lake.
‡The marsh nearly went dry in late summer and fall.

1939 may be attributed both to crowding and to drouth; predation remained negligible, but intraspecific and miscellaneous losses were severe, and skin disease was perhaps as prevalent as it had been in 1936.

DISCUSSION

There is assuredly very much about population phenomena of prolific higher vertebrates that, at present, we can hardly begin to appraise. Compensatory trends in both reproductive rates of the adults and loss rates of the young illustrate the dangers of assuming that numerical and percentage values established for a mortality factor are necessarily a valid measure of population significance of that factor.

Even when the skin disease exerts its maximum influence as a population depressant, as toward the end of the muskrat breeding season, we may by no means be sure that any material proportion of the young which die of the infection would survive in its absence.

SUMMARY

During the summers of 1935, 1936 and 1938, thirty-five, or 9.6 per cent, of 364 litters of muskrats handled on, or in the vicinity of, Round Lake, a northwest Iowa marsh, suffered from skin disease attributable chiefly to *Trichophyton mentagrophytes*. Evidence from other localities, where less intensive studies of young animals were carried on between 1934 and 1940, suggests that the disease may be wide spread, at least within the boundaries of the state. Only young muskrats less than 2 months old appeared visibly affected, infections being invariably fatal to those showing gross manifestations before the age of 2 weeks. Ninety-eight, or 73.1 per cent, of 134 members of infected litters were recorded as contracting the ailment and, of the 98, 90, or 91.8 per cent, apparently died. In general, incidence and severity of infection alike rose as the breeding season (mid-April to late August) of the muskrats progressed. The population significance of the disease, however, was conditioned by intercompensatory trends both in reproductive rates of the adults and in loss rates of the young. Not only were some losses of young—from disease as well as from predation and miscellaneous agencies—offset by prolongation of breeding and production of extra litters, but, under given circumstances, losses of young through intraspecific friction had ways of increasing about in proportion to the extent that other types of losses diminished.

The Effect of Artificial Cryptorchidism on the Prostate Gland of Dogs*

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BILATERAL CASTRATION causes failure of normal development of the prostate gland in puppies and atrophy of the prostate gland in adult dogs.[1] We also have observed failure of normal development of the prostate gland in two dogs in which both testes had failed to descend from the abdomen (congenital abdominal cryptorchids). However, in nine dogs in which only one testis had failed to descend (unilateral cryptorchids), the prostate gland developed normally. Because of the foregoing observations, we wished to know if a normal or hypertrophied prostate gland would undergo regression and atrophy in surgically produced cryptorchid dogs.

Dogs 5 or more years of age having varying degrees of hypertrophy of the prostate gland were selected for these experiments. The condition and approximate size of the prostate gland were noted. The uniformity and size of the testes were noted at the time of operation.

Observations made on nine congenital unilateral cryptorchids indicated that the presence of one testis in its normal situation is sufficient to insure normal development and sustenance of the prostate gland. Therefore, we surgically made unilateral cryptorchids only. In each instance the one testis and its attached spermatic cord were placed in the abdomen and the other testis was taken for study at the time of operation or in other instances allowed to remain in the scrotum until the time of necropsy.

METHODS

The surgical procedures were simple. The dogs were anesthetized with ether and then placed in a dorsal recumbent position. With sterile technic the incision was made immediately proximal and anterior to the scrotum directly over the spermatic cord. In the animals in which one testis was excised and the other was placed in the abdomen, bilateral incisions were made. In the surgically prepared cryptorchids, the testis and its attached appendages were retracted from the scrotum and then were introduced into the abdomen through the inguinal canal and ring. The inguinal ring then was closed with catgut suture. This operation was successful in every instance. Orchectomy was performed by withdrawing the testis from the scrotum through the inguinal canal. The spermatic cord then was ligated and severed. The operations described in later paragraphs were performed on four groups of dogs. Groups 2, 3 and 4 were used as controls for group 1. The dogs were observed for periods varying from 56 to 1,583 days after operation and then were destroyed by inhalation of ether. Necropsy was performed. The prostate gland and any remaining testes were removed, weighed, measured and studied macroscopically and microscopically.

RESULTS

Group 1.—There were seven dogs in this group. The right testis was excised and the left testis and its attached appendages were placed in the abdomen of each dog in this group. Examination at the time of operation revealed the two testes of each dog to be of approximately equal size. The right testis in dogs 3 and 6 showed tumors on examination of cross sections of these testes. The right testis from dog 3 contained a carcinoma of the seminoma type and the right testis from dog 6 contained an interstitial cell adenoma.

No changes in either the size or condition of the prostate glands could be detected by digital palpation. The dogs were destroyed 270 to 798 days after operation and necropsy was performed. Hypertrophy of the prostate gland and atrophy of the cryptorchid testis were noted in each animal (table I). Microscopic examination of the prostate gland revealed the enlargement to be due to benign adenomatous hypertrophy (fig. 1). The atrophy of the testis

*From the Division of Experimental Medicine, Mayo Foundation.

[1]Schlotthauer, C. F., and Bollman, J. L.: The prostate gland of the dog. Cornell Vet., xxvi (Oct. 1936), pp. 343-350.

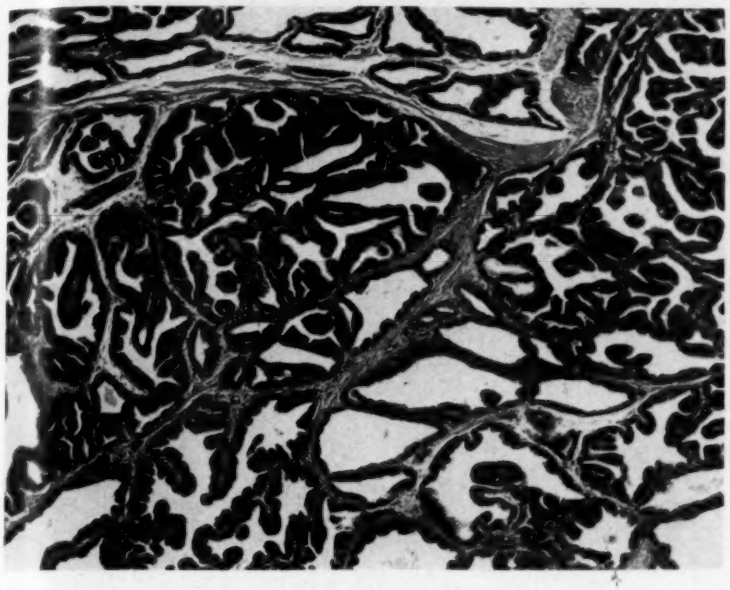

Fig. 1. Benign adenomatous hypertrophy of prostate gland after placement of left testis in abdomen and removal of right testis (x 55).

affected chiefly the tubular elements. Only a single layer of cells remained in each tubule (fig. 2). Nests of normal appearing interstitial cells were noted in the intertubular spaces. They were present in approximately normal number.

Group 2.—There were four dogs in this group. The left testis and its attached appendages were placed in the abdomen of each of these dogs. The right testis was left in its normal situation in the scrotum. Examination at the time of operation revealed the two testes in each animal to be of approximately equal size and essentially normal.

The prostate gland apparently remained unchanged in all of the dogs in this group, but it was thought that the testis remaining in the scrotum enlarged slightly after the operation in every instance. The dogs were destroyed after 230 to 679 days. The prostate gland and both testes were removed at

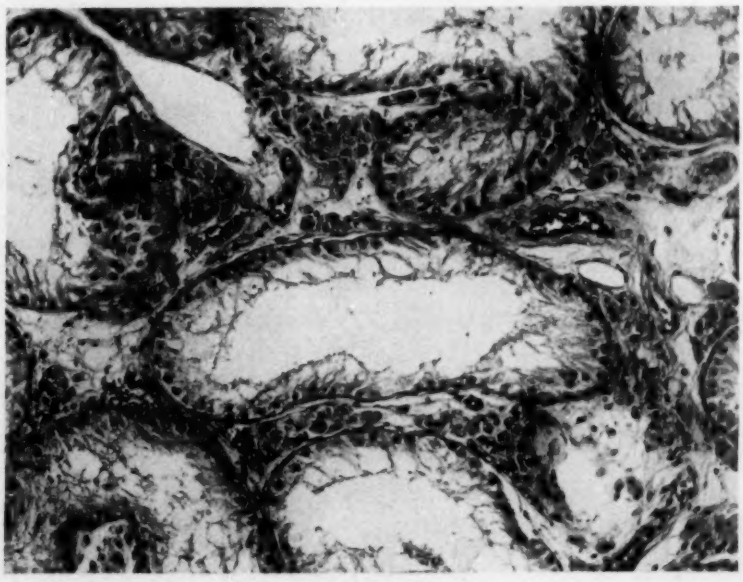

Fig. 2. Atrophy of testis after placement in abdomen (x 120).

Fig. 3. Atrophy of prostate gland after bilateral castration (x 55).

necropsy, weighed and measured. Varying degrees of hypertrophy were observed in the prostate gland and no microscopic evidence of atrophy of the prostate was found in the sections made. The marked variation in the size of the right and left testes was noted (table II). The right testes appeared hypertrophic, whereas the left testes were atrophic. Microscopic examination of the atrophic testes revealed marked atrophy of the tubular elements. The interstitial cells were about normal. This tendency for the scrotal testis to hypertrophy has been noted in congenital unilateral cryptorchids also.

Group 3.—There were two dogs in this group. The right testis was excised from each animal and the left testis was left in its normal situation in the scrotum. Examination at the time of operation revealed the two testes of each animal to be approximately equal in size and essentially normal.

The prostate gland of each dog remained grossly unchanged after operation. One of the dogs was destroyed after 723 days and the other after 1,583 days. The prostate gland and remaining left testis were removed at necropsy, weighed and measured. Hypertrophy of the prostate gland and left testis was noted in each instance (table III). This was confirmed by the microscopic appearance of both tissues.

Group 4.—There were four dogs in this group. Both testes were excised in each of them. Examination of the testes at the time of operation revealed variation in their size in dogs 14 and 17. Gross examination of cross sections of all testes from this group revealed tumors in both testes of dogs 16 and 17. Microscopic examination of the tumors showed them to be interstitial cell adenomas similar to the tumor noted in the right testis of dog 6. Microscopic examination of the testes from dog 14 revealed hypertrophy of the interstitial cells and atrophy of the tubules in the right testis and general atrophy of the left testis. The testes from dog 15 were essentially normal.

Rapidly progressive atrophy of the prostate gland was noted in all of the dogs in this group. They were destroyed 56 to 83 days after operation. The prostate gland of each animal was removed at necropsy, weighed and measured (table IV). The prostate glands were small and firm. Microscopic examination revealed atrophy of the epithelial elements. The acini were obliterated in some areas (fig. 3).

COMMENT

It was apparent that the surgically produced cryptorchid testis retained its ability to sustain the prostate gland. The interstitial cells of the testis were not affected noticeably; this observation tends to sup-

TABLE I—Left Testis Placed in Abdomen, Right Testis Remained in Scrotum

Dog	Wt. (Kg.)	Right Testis Wt. (Gm.)	Left Testis in Abdomen		Prostate	
			Days	Wt. (Gm.)	Diam. (Cm.)	Wt. (Gm.)
1	23.6	15.0	270	9.0	2.5	9.0
2	20.5	13.6	504	5.5	4.6	45.0
3	21.2	21.0*	798	8.0	3.5	16.5
4	14.3	17.5	740	8.0	5.0	22.0
5	13.2	12.0	581	2.8	3.0	12.0
6	11.7	7.6*	764	6.8	4.8	40.0
7	20.0	16.0	798	9.5	3.0	13.0

*Tumors present.

TABLE II—Left Testis Placed in Abdomen, Right Testis Removed

Dog	Wt. (Kg.)	Days	Right Testis Wt. (Gm.)	Left Testis Wt. (Gm.)	Prostate	
					Diam. (Cm.)	Wt. (Gm.)
8	9.7	314	9.7	2.5	3.0	11.5
9	19.8	236	17.0	5.5	2.5	8.5
10	18.2	230	15.5	5.0	2.5	7.0
11	9.2	679	10.0	2.4	3.0	8.6

TABLE III—Right Testis Removed, Left Testis Remained in Scrotum

Dog	Wt. (Kg.)	Right Testis Wt. (Gm.)	Left Testis in Scrotum		Prostate	
			Days	Wt. (Gm.)	Diam. (Cm.)	Wt. (Gm.)
12	19.2	18.5	723	21.5	3.5	15.0
13	15.2	11.0	1,583	22.0	3.5	13.5

TABLE IV—Bilateral Castration

Dog	Wt. (Kg.)	Right Testis Wt. (Gm.)	Left Testis Wt. (Gm.)	Days After Operation	Prostate	
					Diam. (Cm.)	Wt. (Gm.)
14	22.2	24.0*	7.0	56	2.8	8.0
15	17.5	16.0	15.5	58	3.4	5.5
16	17.2	9.0*	8.5*	83	3.0	7.8
17	27.2	5.0*	14.5*	77	2.8	7.0

*Tumor present.

port the hypothesis advanced by Lower[2] that the interstitial cells of the testes elaborate the male sex hormones that cause normal development of the prostate gland. It should be noted that in our experiments normal, well-developed testes were moved to the abdominal cavity and only the tubular structures were altered subsequently. The failure of the prostate gland to develop in the congenital abdominal cryptorchid dog is probably due to the associated failure of testicular development.

Huggins and Clark[3] found that injections of testosterone propionate would cause in time increased secretion and essentially normal redevelopment of the prostate in castrated dogs. They also found that injections of estrogen would cause depression of prostatic secretion in normal dogs and shrinkage of the size of the gland. This change they attributed to depression of production of male hormone, because the changes brought about in the prostate by larger injections of estrogens produced a squamous metaplasia in the posterior lobe and a decrease in the height of the acinar epithelium different from that of the gland after castration. They also were unable to balance completely the effects of injections of androgen by administration of estrogens. We have not found any changes in our cryptorchid dogs which would suggest overactivity of estrogens; in fact, the normal appearance of the gland indicates that sufficient androgens are being produced to maintain the prostate.

[2]Lower, W. E.: The endocrine influence on the male sex organs. New England J. Med., ccIii (April 27, 1933), pp. 878-886.

[3]Huggins, C., and Clark, P. J.: Quantitative studies of prostatic secretion. II. The effect of castration and of estrogen injection on the normal and hyperplastic prostate glands of dogs. J. Exp. Med., lxxii (Dec. 1, 1940), pp. 747-762.

SUMMARY

Bilateral castration causes retrogressive changes and atrophy of the prostate gland in adult dogs. Unilateral castration does not appear to influence the prostate gland. Transplantation of one testis to the abdominal cavity, with or without removal of the other testis, also seems to be without effect on the prostate gland. In these experiments normal testes were placed in the abdominal cavity and the subsequent degenerative changes involved only the tubular cells; no changes of the interstitial tissue were observed even after as long as 798 days. It appears probable that the interstitial cells of the testis, even when placed in the abdomen, secrete sufficient male hormone to maintain the prostate gland in normal condition.

A Filtrable Virus, the Cause of a Respiratory-Nervous Disorder of Chickens*

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DURING THE EARLY months of 1940, young growing chickens concurrently affected with a nervous disorder and a respiratory disease frequently of a severe type were brought to the Poultry Pathological Laboratory at Petaluma from several different ranches in northern California. Most of the specimens examined were from 3 to 10 weeks of age. A brief discussion of the nervous disorder was included in the twenty-first annual report of the State Department of Agriculture.[1]

In a communication to poultrymen, Beach[2] called attention to the condition and reported the results of studies that had been made. The respiratory disease in these early outbreaks was assumed to be infectious bronchitis, despite the fact that in previous years in this region the latter had most commonly affected chicks under 3 weeks of age. The nervous disorder was not then identified. Occasionally, chicks brought to the laboratory showed nervous symptoms alone, and there was no apparent evidence of a respiratory involvement. Inquiry usually revealed, however, that these chicks were from flocks which had just recovered from a respiratory disease.

The nervous disorder in combination with the disease which appeared to be infectious bronchitis became widely distributed among flocks in parts of northern California during the summer and fall of 1940, and for more than a year it has been one of the conditions diagnosed most frequently. This laboratory diagnosed 58 outbreaks in 1940 and 98 in 1941 to October 1.

Many poultrymen now regard the disease showing this combination of symptoms as the most serious of those affecting their growing chickens. Incidence of cases of the nervous disorder in affected flocks ranged from 1 to 45 per cent. Most of the birds showing the nervous condition either died or were destroyed. Nearly 100 per cent of the birds in many affected flocks showed respiratory symptoms. The mortality was sometimes as high as 15 per cent even though few birds had developed the nervous condition. The average mortality in flocks showing both respiratory and nervous symptoms was probably between 5 and 10 per cent. Some poultrymen reported that they were much more concerned about the economic loss resulting from a marked unthriftiness of the survivors than they were about the death loss. Many such unthrifty fowls were examined at the laboratory and found to be apparently free of other diseases.

While studying the nervous disorder in chickens, Beach[2] made an investigation of the possibility that this condition might be due to an invasion of the brain by the chick-bronchitis virus. Efforts to prove that this nervous disorder is of infectious origin have thus far been unavailing, he reported. Consideration also was given to the possibility that the condition is of dietary origin. Feeding experiments were in progress at the time of his report.

The alarming death rate in flocks of older birds in which there were relatively few cases of the nervous disorder first caused us to suspect that we were dealing with a new respiratory disease. Infectious bronchitis was the only condition it resembled, but in previous years the disease recognized as infectious bronchitis in this region chiefly affected young chicks and appeared to be the same as the condition described by Schalk and Hawn[3] in 1931, by Bushnell

*From the Poultry Pathological Laboratory, California State Department of Agriculture.

[1]Report of the Poultry Pathological Laboratory, Petaluma. State of California, Dept. Agr. Bul., xxix (1940), pp. 367-368.

[2]Beach, J. R.: A nervous disorder in chickens. Poultry News, xviii (1941), p. 13.

[3]Schalk, A. F., and Hawn, M. C.: An apparently new respiratory disease of baby chicks. J.A.V.M.A., lxxviii (1931), pp. 413-422.

and Brandly[4] in 1933 and by Beach and Schalm[5] in 1936, and by other investigators later. An important development that tended to strengthen the possibility that a new respiratory disease had become endemic here occurred when an increasing number of poultrymen reported that their flocks of growing chickens seemingly had twice become affected with infectious bronchitis, with usually the severe attack following the milder one. In two instances it was discovered that we had diagnosed infectious bronchitis in a group of chicks at an early date and then a few weeks later had diagnosed the disease again in the same group. These developments suggested further that although the nervous disorder might not be caused by the virus of infectious bronchitis, it could be caused by some other virus that affects also the respiratory system of the chicken.

Our work now indicates that the nervous disorder and the accompanying respiratory symptoms are both manifestations of one disease entity. This is distinct from the disease which for some years was recognized by us as infectious bronchitis. The new condition will hereinafter be referred to as a respiratory-nervous disorder.

CHARACTERISTICS OF THE DISEASE

In artificially infected chickens the incubation period was found to vary between two and six days, with an average of four days. Field observations revealed that the respiratory-nervous disorder nearly always ran a considerably more protracted course, both in the flock and in the individual bird, than did the disease recognized here as infectious bronchitis. Disregarding the nervous symptoms, which sometimes lasted indefinitely, affected chicks often showed respiratory symptoms for three or four weeks and the flock as a whole for five or six weeks. Sometimes the rate of spread in a flock was slow. Frequently, many birds in a flock remained healthy for several days after the first deaths occurred. Some birds never showed visible symptoms of any kind. In reviewing outbreaks on some ranches with several pens of birds of the same age, it was noted that some pens had been very lightly affected while others suffered severely. In some instances nervous symptoms did not occur until toward the end of the outbreak, but usually they were observed a day or two after it first became evident that the flock was affected with a respiratory ailment. In general it may be stated that the younger the chicks, the more susceptible they were to the nervous form of the disease. The death losses started early in the outbreaks and continued for several weeks thereafter.

As indicated previously, most of the outbreaks were in fowls that were in the age range of 3 to 10 weeks. A few outbreaks in which nervous symptoms were displayed were observed in younger chicks and in laying hens past 1 year of age. A respiratory disturbance commonly occurring alone in laying hens in this region during the past year was tentatively diagnosed as infectious bronchitis because of its apparent similarity to a disease in semimature and older chickens in Rhode Island identified by Delaplane and Stuart[6] as infectious bronchitis. There now appears to be good reason to suspect that some of the cases previously diagnosed as infectious bronchitis were affected with the respiratory-nervous disorder.

Respiratory and General Symptoms.—In typically affected growing chickens, the first symptoms usually noted were droopiness, occasional sneezing or shaking of the head and husky voice. A few birds gasped for breath. Breathing was apt to become labored in all of them as the disease progressed. Affected birds then often became paralyzed in the legs or exhibited other nervous symptoms. At the height of an outbreak, body temperatures were between subnormal and 111 F. The higher temperatures were in birds sick but a day or two. Death resulting from an acute attack was usually preceded by a period of coma lasting a few hours, during which body tem-

[4]Bushnell, L. D., and Brandly, C. A.: Laryngotracheitis in chicks. Poultry Sci., xii (1933), pp. 55-60.

[5]Beach, J. R., and Schalm, O. W.: A filterable virus, distinct from that of laryngotracheitis, the cause of a respiratory disease of chicks. Poultry Sci., xv (1936), pp. 199-206.

[6]Delaplane, J. P., and Stuart, H. O.: Studies of infectious bronchitis. Rhode Island Agr. Exp. Sta. Bul. 273 (1939).

peratures dropped below 90. Birds which passed the crisis soon became bright in appearance even though they still showed evidence of respiratory distress or of a nervous disorder.

Nervous Symptoms.—Nervous symptoms were of three general types: muscular incoördination involving particularly the neck and leg muscles, constant tremor of the body and paralysis of the legs. Birds that were able to walk, eat and drink went through many waste motions. Walking backwards or in circles and a continual twisting of the head and neck were of common occurrence. A quick tremor of the body and a jerking motion of the head and legs with each exhalation were more often manifested by birds unable to stand. It was not unusual, however, for a bird not paralyzed in the legs to show a slight body or head tremor for an indefinite period following recovery from the respiratory phase. Muscular incoördination involving the muscles of the neck was another manifestation which apparently lasted indefinitely. Birds paralyzed in the legs lived for an extended period too, provided they had access to feed and water. In most flocks paralysis of the legs was the most commonly observed nervous symptom. Partial or complete recovery from the nervous afflictions occurred in a few birds.

The nervous symptoms displayed by some of the affected birds had a marked resemblance to symptoms observed by Jones[7] and by Van Roekel and coworkers[8] in chickens affected with infectious avian encephalomyelitis.

Lesions.—A lesion quite consistently found in the respiratory system of newly affected chickens was a cloudiness of the air sacs. As the disease progressed, the air-sac membranes became edematous, yellowish gray and almost opaque. Within the air sacs of chickens affected for several days, a purulent exudate or particles of yellow, caseous material commonly adhered to the membranes, particularly at their attachments. A frothy, colorless, serous fluid was frequently seen in the body cavity. Occasionally the mesentery, which may have been covered with the froth, had a slightly cloudy or turbid appearance. Edema or congestion of the lungs and frothy mucus in the bronchi and lower portion of the trachea were present in a considerable number of acute cases. Visible inflammation of the bronchi and trachea and the presence of caseous material here and in the sinuses were of less frequent occurrence. It is suspected that lesions of this type, when found, may be indicative of a concurrent respiratory disease. The more clearly perceptible lesions associated with the respiratory-nervous disorder were confined primarily to the portion of the respiratory system lying below the bronchi. Otherwise there was little to distinguish the lesions of this disease from those of the disease recognized here as infectious bronchitis.

Microscopic or gross lesions in the brain and nervous system were not detected. We expect, however, to make a further search for lesions which might cause the nervous symptoms.

TRANSMISSION EXPERIMENTS

Infected air-sac membranes from three 5-week-old chickens which had shown both the respiratory and nervous symptoms were ground in a sterile mortar. The macerated tissue, after being mixed with approximately 4 cc. of physiologic salt solution, was injected intranasally into two chickens 6 weeks of age. Two other chickens of the same age and from the same source were held as controls. These two birds were kept in a cage located within a few feet of cages in which infected birds were held, and though no precautionary measures were used to prevent accidental infection in the former, they did not contract the disease until after they were artificially infected several weeks later. The two inoculated birds became paralyzed in the legs and showed evidence of respiratory distress on the fifth and sixth days, respectively, following inoculation. Both were destroyed on the same day that the second bird became paralyzed and they were found to have cloudy air sacs. Infective material recovered from the air sacs was used to inoculate other birds. Eleven serial passages of this material have now been made through chickens ranging in age from 4 days to 3 months. Rapid passage of the infective agent through several lots of chickens resulted in a shortening of the

[7] Jones, E. E.: Epidemic tremor, an encephalomyelitis affecting young chickens. J. Exp. Med., lix (1934), pp. 781-798.

[8] Van Roekel, H., Bullis, K. L., and Clarke, M. K.: Preliminary report on infectious avian encephalomyelitis. J.A.V.M.A., xciii (1938), pp. 372-375.

incubation period from an average of 5 days to an average of 3 days.

The serial-passage birds have not only made available a constant supply of infective material, but have afforded an excellent opportunity for studying the disease. Five or more chickens have been used for each passage, and inoculations have been made every 4 to 6 days. Since it was found that the infective agent was present in the trachea and lungs of newly affected birds, these tissues in addition to the air-sac membranes were used as inoculum in later work. After being placed in a small amount of the saline solution, the tissues were macerated as before and then pressed through gauze. Additional small amounts of saline solution were added as required. Nearly all passage birds received both intranasal and intratracheal injections of this material, the dosage not exceeding 0.2 cc. in the latter instance.

Results.—Attempts to infect chickens by either intratracheal or subcutaneous inoculation alone or by placing a gelatin capsule containing the infective agent directly into the crop were all successful. The method of inoculation seemed to have little effect on the length of the incubation period. That the disease may be transmitted by pen contact was demonstrated when healthy chickens contracted the disease within a few days after they had been placed with artificially infected chickens. The disease was usually of a milder type, however, in the naturally infected birds than in the artificially infected pen mates. It seems probable that the usual method of transmission is by the ingestion of food and water contaminated with the excretions of diseased fowls.

Two 5-week-old ducks, two 6-week-old pigeons, two 8-week-old turkeys, and two 4-week-old rabbits, all of which had never shown evidence of disease at any time, were refractory when tested. Refractory also were five 2-year-old chickens whose past history was not known. All were inoculated either intranasally or intratracheally.

FILTRATION EXPERIMENTS

Although there had been little effort to determine whether or not the disease was of bacterial origin, it was decided that its nature would warrant the making of filtration tests without further delay.

Before the experiments were begun, the porosity of each of the Berkefeld filters that were to be used was tested according to the method of Ward and Tang,[9] by which is determined the amount of water that will flow through the filter in five minutes at a negative pressure of 10 cm. of mercury. The most porous V filter passed 82 cc. of water, and the least porous W filter passed 26 cc. Other details concerning the filters are presented in table I.

Material to be filtered was obtained in the manner already described. For each filtration test a suspension was made of the macerated air-sac membranes, lungs and tracheas from two or three affected chicks 10 days to 3 weeks of age. Control birds were inoculated with a few drops of the unfiltered suspension. Before filtration through the candle, the suspension was passed through filter paper and mixed with 0.25 cc. of a 24-hour-old broth culture of *Serratia marcescens* (*Bacillus prodigiosis*). The volume of fluid to be used for the test was then adjusted to 3.5 cc. by the addition of physiologic salt solution. A culture on agar was made from the fluid at this time. After the entire amount of fluid had been taken up by the filter, an additional 1 to 2 cc. of saline solution was placed on the candle for the purpose of forcing a larger volume of the first lot of fluid through the filter. It was thought that a filtrable infective agent, if present, might be obtained in greater concentration by this means than would be possible if the additional amount of saline solution were added to the suspension beforehand. Although the candles were of the 2½-in. size, there was no difficulty in passing approximately one half of the small amount of fluid used. Fluid lying in the mantle was picked up repeatedly with a long dropper pipette and allowed to flow over the entire surface of the candle. Filtration was done at a negative pressure of 40 to 80 cm.

One fourth of the filtrate was removed from each filter flask with a sterile pipette, transferred to a tube of beef broth, and incubated for two weeks to test for the presence of *S. marcescens*. The filtrates all proved to be free of *S. marcescens* or other aerobic bacteria.

From three to five chicks, 10 days old, were inoculated with the remaining filtrate in each flask. Individual dosage was from 0.5 to 1 cc. As indicated in table I, three different strains of the infective agent were used in the filtration tests. Strain L was the passage strain which had proved to be invariably potent, and strain B and strain G were acquired more recently from badly affected birds submitted for examination.

Results.—In table I is presented a summary of 15 filtration experiments in which

[9]Ward, H. K., and Tang, F.: A note on the filtration of the virus of herpetic encephalitis and of vaccinia. J. Exp. Med., xlix (1929), pp. 1-4.

TABLE I—Summary of Filtration Experiments

Filters			Filtrations		
Grade	Number Used	Filter No. & H_2O Test	Strain of Virus	Number Attempted	Number of Filtrates Infective
V	3	V-1 (New) 80 cc.	L	2	2
		V-2 (New) 82 cc.	L	1	1
		V-3 (Used) 27 cc.	B	1	1
N	2	N-1 (New) 41 cc.	L	1	1
			B	1	1
		N-2 (Used) 35 cc.	L	3	3
			G	1	1
W	2	W-1 (New) 31 cc	L	1	1
			B	1	1
		W-2 (Used) 26 cc.	L	2	2
			G	1	1

all grades of Berkefeld filters and three strains of the disease were used.

The chicks inoculated with the filtrates developed the respiratory-nervous disorder as quickly as did the inoculated control birds of the same age, and there was no apparent difference in the intensity of the disease in the two groups. All inoculated birds developed respiratory symptoms and, with few exceptions, nervous symptoms within seven days after inoculation. Nearly all died of the disease. Both groups probably received far more of the infective agent than was needed to induce infection. No attempt was made to determine if there was any adsorption of the infective agent by the filters.

Five or more uninoculated chicks from the same source and of the same age as the inoculated chicks were held for at least two weeks following each inoculation of test birds. In not a single instance did any of the uninoculated control groups become accidently infected, and all proved to be susceptible to the respiratory-nervous disorder when inoculated later.

The results of these filtration experiments establish the fact that the respiratory-nervous disorder is caused by a filtrable virus which is capable of passing all three grades of Berkefeld filters.

Cross-Immunity Experiments

After a study had been made of several different outbreaks of apparently typical infectious bronchitis in brooder chicks, two different strains of the infective agent were selected for the purpose of conducting cross-immunity tests. These strains were designated as M and J. Filtrability of the infective agent was established by passing it through Berkefeld filters. Filtrates of M and J strains of virus were inoculated intranasally and intratracheally into chicks 5 to 7 days old. The chicks receiving strain J were held in a building several miles from the building in which the chicks receiving strain M were housed, and both groups were in buildings entirely separate from the one in which the chickens affected with the respiratory-nervous disorder were held.

The incubation period in inoculated chicks ranged from 1 to 4 days, and the course was 6 to 18 days. Upon recovery, the chicks were reinoculated with an unfiltered virus of the same strain with which they had been first inoculated and proved to be refractory. They were then inoculated with either strain L or B virus of the respiratory-nervous disorder. All groups

TABLE II—Summary of Cross-Immunity Experiments with Three Strains of the Respiratory-Nervous Disorder and Two Strains of Infectious Bronchitis

Original Strain of Virus	Strain of Virus for Susceptibility Test	Number of Trials	Number of Chickens Tested	Number Susceptible
Inf. bronchitis — M	Resp. nervous — L	1	2	2
Inf. bronchitis — J	Resp. nervous — L	4	15	15
Inf. bronchitis — J	Resp. nervous — B	1	2	2
Resp. nervous — L	Inf. bronchitis — J	4	9	3
Resp. nervous — G	Inf. bronchitis — J	1	7	5
Inf. bronchitis — J	Inf. bronchitis — M	1	5	0
Resp. nervous — L	Resp. nervous — B	3	9	0

developed the disease as severely as did the inoculated control group, and the disease manifested itself in the usual manner, with some birds becoming paralyzed in the legs or showing other nervous symptoms and all showing distinct evidence of a respiratory involvement.

Cross-immunity experiments in which groups of chickens refractory to the respiratory-nervous-disorder virus were tested for susceptibility to infectious bronchitis did not give as definite results. There were available for these tests relatively few survivors from groups inoculated with the virus of the respiratory-nervous disorder, and because of the long period of convalescence they were considerably older than chickens usually are when they develop infectious bronchitis in its most severe form. Fifty per cent of the birds that were demonstrated to be refractory to the respiratory-nervous-disorder virus were still susceptible to infectious bronchitis when tested. The only manifestations in the affected birds were a change in the voice and the presence of abnormal amounts of clear, watery mucus in the nasal passages. It is possible that some of the other birds which were not considered to be affected following their inoculation with the virus of infectious bronchitis actually did develop the disease in even a milder form.

In another experiment, chickens which had recovered following artificial infection with the L strain of the respiratory-nervous-disorder virus were found to be refractory to the B strain of the same virus. Likewise, chickens which had recovered following artificial infection with the M strain of the infectious bronchitis virus were found to be refractory to the J strain of the same virus.

In order to determine whether or not the respiratory-nervous disorder and the disease which was assumed to be infectious bronchitis in these studies were in any way related to infectious laryngotracheitis, seven chickens demonstrated to be immune to the L strain of the respiratory-nervous disorder and to the J strain of infectious bronchitis were inoculated intracloacally with a commercial laryngotracheitis vaccine. All showed good "takes" on the fourth and fifth days following inoculation, thus showing that neither of the two respiratory diseases of filtrable-virus origin which previously had been under consideration was laryngotracheitis. Four of the seven birds were later tested for susceptibility to infectious coryza of the type caused by *Hemophilus gallinarum*. A typical form of the disease was produced in each of the birds.

The results of the cross-immunity experiments indicate that the respiratory-nervous disorder is distinct from the respiratory diseases that have been enzoötic among chickens here for a number of years. A summary of the cross-immunity experi-

ments with the three filtrable-virus diseases is given in table II.

SUMMARY AND CONCLUSIONS

1) A respiratory-nervous disorder of chickens has become increasingly important since it first appeared in northern California flocks early in 1940. Most of the birds which became affected were 3 to 10 weeks of age. Some outbreaks have been observed in younger chicks and in mature hens.

2) The respiratory phase of the disease was similar to infectious bronchitis, and it was difficult to differentiate between the two on the basis of symptomatology. It is probable that a flock may become affected with both diseases at the same time.

3) Nervous symptoms of three general types usually were observed in each affected flock within a few days after the birds first began to show evidence of a respiratory disease. These were muscular incoördination involving particularly the neck and leg muscles, constant tremor of the body and paralysis of the legs.

4) The infective agent was present in the air sacs, lungs and tracheas of the affected chickens and produced the disease in other chickens when inoculations were made intranasally, intratracheally, subcutaneously or directly into the crop. The disease was transmitted by pen contact.

5) Experiments have shown that infective material extracted from the air sacs, lungs and trachea when suspended in physiologic salt solution and passed through Berkefeld V, N or W filters produced the disease. These results demonstrated that the disease is caused by a filtrable virus.

6) Studies showed that chickens refractory to infection with the virus of infectious bronchitis were susceptible to the virus of the respiratory-nervous disorder. Chickens refractory to the respiratory-nervous disorder were susceptible to infectious bronchitis in 50 per cent of the cases tested.

7) Chickens that were immune to the respiratory-nervous disorder and to infectious bronchitis were shown to be susceptible to the virus of infectious laryngotracheitis and to coryza caused by *Hemophilus gallinarum*.

8) The results of the cross-immunity experiments indicate that the virus of the respiratory-nervous disorder is distinct from the virus of infectious bronchitis and from that of infectious laryngotracheitis.

Further Observations on a Blood Protozoan of Turkeys Transmitted by Simulium Nigroparvum (Twinn)*

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SINCE THE REPORT by Johnson *et al.*[1] concerning the transmission of, and stages in the life cycle of, a blood protozoan of turkeys that resembles *Leucocytozoon smithi*, further work has been done with the hope of determining both asexual and sexual stages in the complete life cycle of this parasite. From the evidence presented by these workers, it was concluded that (a) sporozoites enter the blood of the turkey, during the bite of infected simuliids of the species described and, in the blood plasma, first change to immature gametocytes which grow to maturity, and in this stage may remain for several months; (b) both male and female gametocytes occur, which can be distinguished readily on the basis of their tinctorial properties, and when these are taken into the stomach of the fly, they develop into macro- and microgametes, respectively; (c) after fertilization the resulting zygote uncoils to become a motile oökinete; (d) sporozoites appear in the salivary glands, although no stage of the parasite between the oökinete and the sporozoite was identified with certainty. The apparent dissimilarity in schizogony of this parasite with *L. anatis* described by O'Roke[2] and with *Haemoproteus columbae* described by Aragao and by Adie as reported by Wenyon[3] demands further investigation, especially since the cycle reported does not include certain schizogonous stages described for other species of this genus.

The investigation reported herein was outlined to determine (1) if any schizogonous stages of the parasite occur in any tissue of the infected turkey, (2) what blood cell of the turkey, if any, is entered by the sporozoite, or by the gametocyte, and acts as the host cell, and (3) if an oöcyst stage occurs in the infected fly.

REVIEW OF LITERATURE

According to Wenyon,[3] the name Leucocytozoon was first given to certain unpigmented parasites seen by Danilewsky in the blood of birds. He suggested this name because he considered them to be parasitic within leucocytes and, though this may not actually be the case, the name still stands. It is claimed that members of this genus are parasitic in certain cells of the blood, which contain no hemoglobin. Wenyon states further that the observations of Moldovan on *L. zeimanni* indicate that the asexual reproduction takes place in the internal organs of the bird, probably in the endothelial cells of the vessels, as in the case of Haemoproteus. In his own work, however, no asexual reproduction phases were observed in guinea fowls, though these were specially looked for. Schaudinn on the other hand, considered the entire structure as the parasite and did not think that any host cell was involved.

Most workers feel that the asexual cycle of leucocytozoa in general follows closely the cycle described by Aragao (Wenyon) for *H. columbae* in the pigeon. Briefly, this is supposed to take place as follows: Sporozoites are introduced by the bite of *Lynchia maura*, the transmitting host. They then are supposed to enter endothelial cells of the various organs, although it is admitted that the actual behavior of the sporozoites after injection into the pigeon has not been followed. In these endothelial cells the sporozoites develop into schizonts. The multinucleated cytoplasmic bodies, or cytomeres, that are formed divide into enormous numbers of minute merozoites. These, when liberated into the blood stream, develop into gametocytes and enter erythrocytes of the pigeon.

In more recent work, with *L. simondi* of ducks, Dr. C. G. Huff of the University of Chi-

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*From the Section of Animal Pathology, Virginia Agricultural Experiment Station; presented before the Section on Poultry at the seventy-eighth annual meeting of the American Veterinary Medical Association, Indianapolis, Ind., August 11-15, 1941.

[1]Johnson, E. P., Underhill, G. W., Cox, J. A., and Threlkeld, W. L.: A blood protozoon of turkeys transmitted by *Simulium nigroparvum* (Twinn). Am. J. Hyg., xxvii (1938), pp. 649-665.

[2]O'Roke, E. C.: A malaria-like disease of ducks, caused by *Leucocytozoon anatis* Wickware. U. of Michigan, School of Forestry and Conservation, Bul. 4 (1934).

[3]Wenyon, C. M.: Protozoology (William Wood & Company, New York, 1926), ii, pp. 883-908.

cago[4] states that he is convinced that the gametocyte grows up in lymphocytes, monocytes and probably in macrophages.

From these reports it is evident that there is not complete agreement among workers in this field as to the host cell entered by the gametocyte, nor has anyone conclusively demonstrated which cell is entered by the sporozoites introduced by the transmitting host.

MATERIALS AND METHODS

The materials studied comprised scores of blood smears stained in various ways so that chromatin particles in any host cell would be recognized. Tissues from 21 turkeys injected with infected simuliids were sectioned at from 5 to 7 μ, including tissues obtained from birds 24 hours after the injection and those from birds after increasing the time by 24-hour periods until a 12-day post-injection period was reached before the bird was killed.

Approximately 200 engorged simuliids were used for the injections and about 50 were sectioned and stained in order to study sporogonous stages in the flies. Moist blood and other tissue smears were fixed in methyl alcohol and stained with Giemsa's stain for the most part. Several, however, were fixed in Schaudinn's fluid and stained with Heidenhain's iron hematoxylin. Tissue sections from turkeys were stained with dilute Delafield's hematoxylin followed with azure II and eosin after fixation in Helly's fluid. Sections containing the parasite also were stained according to Feulgen's method for the detection of nuclear chromatin. The flies were fixed in either Bouin's or Schaudinn's fluid and stained with azure II plus eosin following Delafield's hematoxylin.

EXPERIMENTAL PROCEDURE

Our previous inability to demonstrate any schizogonous stage of the parasite in turkeys prompted us to try exhaustive methods in order to determine whether or not any such stages do occur in tissues of the turkey. In a study of blood smears prepared daily from infected turkeys it was found that gametocytes first appear in the peripheral blood 9 to 13 days after the turkey has been bitten by, or injected with, infected simuliids. A careful study of stained tissue sections from these birds failed, however, to reveal the presence of schizogonous stages.

The possibility that all such stages occur before the appearance of gametocytes in the peripheral blood, while remote, could not be overlooked. In order to determine this, a series of carefully controlled young turkeys were infected artificially and killed for study, beginning with a 24-hour post-injection study which was increased by 24 hours until tissues from infected turkeys obtained after post-injection periods ranging from 24 hours up to 12 days had been obtained. In this work 21 turkeys were used; thus, tissues from two birds from each period were available after the 72-hour period. For this purpose poults removed directly from the incubator and brooded in flyproof cages were used. Daily blood smears from each bird were examined at least two weeks before injecting with infected simuliids to make certain that infection had not taken place. The simuliids used for injection purposes were maintained alive at least 72 hours after engorging on an infected turkey. It was demonstrated previously[1] that sporozoites occur in the salivary glands of *Simulium nigroparvum* 72 to 120 hours after feeding on infected turkey blood.

At the time of autopsy of the infected turkeys, blood smears as well as smears from the spleen, liver and femoral marrow were made in addition to fixing pieces of tissue from the heart, lungs, liver, kidneys, spleen, femoral marrow and, in some instances, brain for histologic study. Beautifully stained sections were obtained, especially with Helly's fixation and staining with dilute Delafield's hematoxylin and azure II plus eosin. These were examined with the aid of Leitz 10 x oculars and 4-mm. objective as well as with a 2-mm. apochromatic objective, careful attention being given to endothelium of vessels in lungs, spleen, kidneys, liver and marrow. In no instance were schizogonous stages observed.

Experimental evidence that schizogonous stages do not occur was obtained from a study of turkeys kept in screened cages for several months. It has been observed for several years that turkeys carrying a large number of gametocytes in their blood in August will invariably lose them, with the result that by January very few, if any,

[4]Huff, C. G.: Personal communication.

can be demonstrated in their blood. If schizogonous or asexual reproduction occurs, some merozoites should develop into gametocytes, which could be detected in the blood of turkeys that were screened away from flies to prevent reinfection from that source. Three infected turkeys were placed in screened cages in August and kept under such conditions for a year. Periodic blood examinations were made every two or three weeks. In each case the parasites gradually diminished in numbers until February, when none could be demonstrated. Continued observations followed by histologic study of tissues failed to demonstrate that parasites were present in July, when the experiment was terminated. Control birds left unprotected began to have gametocyes in their blood in April after being negative for two months. This proves that reinfection takes place rather than recurrence in the peripheral blood as the result of schizogonous development in the tissues.

In this material abundant opportunity was furnished to study the parasite under various conditions. Figures 1, 2, 3 and 4 illustrate various stages of immaturity of the gametocyte. The lateral bar or bars which are considered by some workers to be host-cell nuclei give no staining reactions to indicate that they contain chromatin materials. Their appearance after being stained with supravital dyes, such as neutral red and Janus green, further emphasizes the conclusion that the whole structure constitutes the parasite and that no host cell is involved.

In first studying the blackfly for sporogonous stages we were unable to decide whether or not an oöcyst stage occurred. Most of our difficulty in finding this stage was due to the location of the oöcyst as compared with the location of this stage of malaria organisms in the mosquito. No evidence was found that this occurs on the stomach wall, as in the mosquito. This stage does occur, however, in the stomach wall of *S. nigroparvum* as illustrated in figures 5 and 6. This may be unique for this species. The zygote uncoils in the stomach contents of the blackfly to become a motile oökinete. This vermiform stage migrates into the stomach wall and there rounds up to become the oöcyst stage. The oöcyst stage then ruptures to release the sporozoites, which later find their way to the lumen of the salivary glands.

DISCUSSION

The evidence obtained in these experiments points clearly to the conclusion that the gametocytes in the blood of infected turkeys as described by Johnson *et al.*[1] and referred to as *L. smithi* by other workers[5-7] are present in the blood plasma as such and do not occur within any blood cell. The experimental evidence submitted can be interpreted in no other way. The interpretation by O'Roke[2] as regards *L. anatis* in erythrocytes of ducks does not seem tenable, since it is inconceivable that a cell the size of a duck erythrocyte could contain within it a parasite more than twice its own size. For similar reasons the turkey erythrocyte could not be considered as a host cell for *L. smithi*.

The life span of the erythrocyte in turkeys is undoubtely not much longer, if any, than in human beings, where the average span is from 30 to 100 days. Likewise, Jor-

[5]Skidmore, L. V.: *Leucocytozoon smithi* infection in turkeys and its transmission by *Simulium occidentale* Townsend. Cent. f. Bakt., Abt. I, Orig., cxxv (1932).

[6]Smith, T.: Infectious diseases among poultry. USDA, BAI, Bul. 8 (1895).

[7]Volkmar, F.: Observations on *Leucocytozoon smithi*; with notes on leucocytozoa in other poultry. J. Parasitol., xvi (1930), pp. 24-28.

CAPTIONS TO ILLUSTRATIONS ON OPPOSITE PAGE

1) Earliest stage of the gametocyte encountered, occurring in a hepatic vessel of the turkey (x 1,380).

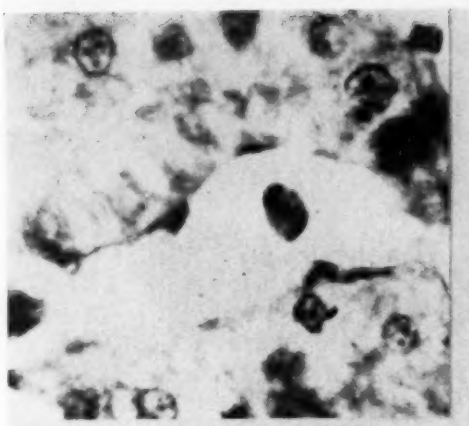
2) A stage somewhat later than that shown in figure 1. Note size compared with turkey erythrocytes (x 750).

3) A still later stage than shown in figures 1 and 2. Compare size with turkey granulocyte in same field (x 750).

4) Three mature gametocytes. Note spindle shape, bilateral bars and size compared with turkey erythrocytes (x 368).

5) Three oöcysts in stomach wall of S. nigroparvum (x 1,380).

6) Oöcyst and oökinete in stomach wall of S. nigroparvum.



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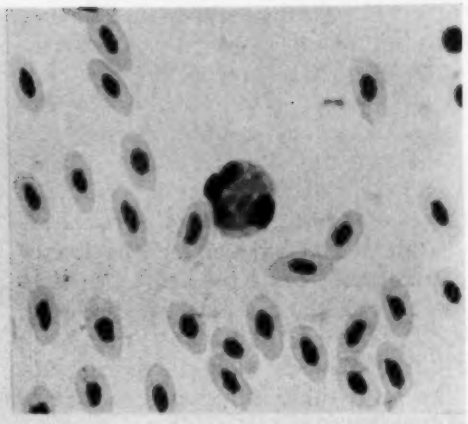

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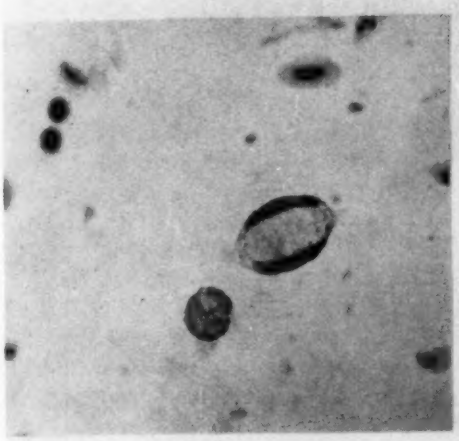

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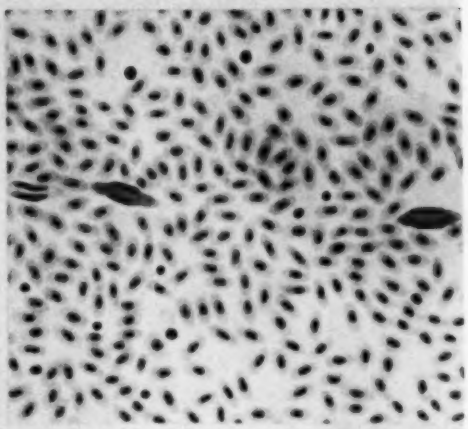

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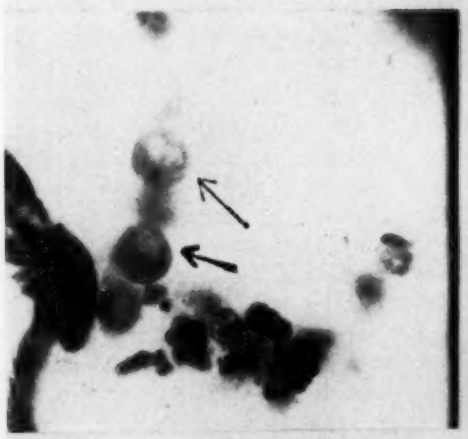

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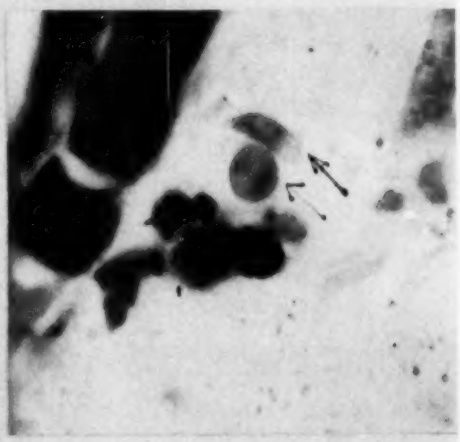

6

dan[8] found the average life tenure of erythrocytes of the active frog to be approximately 90 days. It may be assumed that the life tenure of turkey erythrocytes approximates what has been established for human beings and for the frog. Consequently, it would not appear that the gametocyte stage of this parasite could remain within a cell several months longer than the normal life of the cell. On these grounds, as well as on a morphologic basis, there being no apparent chromatin material in the lateral bars or alleged host-cell nucleus, one is justified in concluding that *L. smithi* does not parasitize turkey erythrocytes.

For some time it was thought that the lymphocyte might be the victim, especially after the various growth potentialities of this cell were considered. Here again, however, this cell may be ruled out on morphologic grounds as stated in connection with the erythrocyte. Less is probably known with regard to the actual life span of lymphocytes than there is about erythrocytes, but the suggestion by Jordan[9] seems to offer a tenable explanation at least for the fate of the enormous number of lymphocytes that daily leave the circulation. He concludes that many of these are filtered out in the bone marrow to act as ancestral cells to other types of blood cells, including erythrocytes, granulocytes and monocytes. On the basis of Yoffey's work, as reference is made to it by Jordan, more lymphocytes are lost daily in the body of the mammal than are present at any one time in the circulating blood. It is stated that Yoffey summarizes his experiments on the dog with the statement that the lymphocytes are replaced 2½ times every 24 hours. With such rapid replacement of lymphocytes it is scarcely conceivable that this cell should, when parasitized, remain in the circulation for several months. Daily blood smears in which lymphocytes can be carefully studied fail to reveal any immature stages of the parasite in these cells. It is only after a period of from 9 to 13 days after infection that the gametocytes can be demonstrated in the peripheral blood.

[8]Jordan, H. E.: Comparative hematology. Handbook of hematology (Paul B. Hoeber, Inc., New York, 1938), ii, pp. 699-862.

[9]Jordan, H. E.: The lymphocytes in relation to erythrocyte production. Anat. Rec., lxxiii (1939), pp. 227-241.

SUMMARY

In histopatholgic studies of tissues from 21 turkeys infected with *Leucocytozoon smithi* and killed 24 hours to 12 days after the injections, schizogonous stages of this protozoan were not found.

From continued observations it is concluded that the gametocytes of this protozoan as observed in the blood of affected turkeys occur in the plasma and not in any host cell.

In a further study of turkeys which were infected with this parasite and in which numerous gametocytes could be demonstated in the peripheral blood for several months, when kept screened away from flies for a year gametocytes did not recur in their blood, while in control turkeys exposed to flies, gametocytes did recur, indicating that asexual reproduction of the parasite did not take place in the turkey.

The oöcyst stage occurs in the stomach wall of *Simulium nigroparvum* and not on the wall, as do malaria organisms in the mosquito.

From this work it appears that the life cycle of *L. smithi* as it occurs in turkeys and in *S. nigroparvum* is as follows: Macro- and microgametocytes are taken into the stomach of these blackflies while engorging on blood from infected turkeys. These develop into macro- and microgametes, respectively, and after fertilization the resulting zygote uncoils in the stomach of the fly to become a motile oökinete which finds its way into the stomach wall where it rounds up to become an oöcyst. The oöcyst, after maturing, ruptures to release sporozoites, which find their way to the lumen of the salivary glands of the fly. When the infected flies bite turkeys, the sporozoites enter the blood stream, where they develop in the plasma into gametocytes.

An Electrophoretic Study of Pullorum-Agglutinating Chicken Serums*

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IN SOME LOCALITIES it has been found that pullets in the first stages of production give apparently nonspecific positive reactions to the agglutination test for pullorum disease. Most of these reactions have shown up in the whole-blood plate test, but a high percentage of such reactors will also give positive reactions in the tube test. These nonspecific reactions are prevalent in certain flocks during the fall months. When such birds are held for two to three months and retested, they usually are found to be negative. An unusually high percentage of conspicuously uniform agglutination reactions should warn the tester not to condemn such reactors without a retest.

Attempts have been made to find the cause of these reactions. Organisms antigenically related to *Salmonella pullorum* carried by chickens may be responsible. It is known that certain members of the Proteus, Shigella, Escherichia and Aerobacter groups, isolated from chickens, are agglutinated by pullorum-agglutinating chicken serums.

Another possible cause of these reactions may be the physico-chemical nature of the blood at this stage of life. The demands upon blood protein are unusually great. The colloidal equilibrium of the blood is constantly changing. Since the antibodies are intimately associated with the globulin fraction, it was surmised that an electrophoretic study of the serums might disclose significant differences in the properties of the serum proteins.

The serum samples were examined in the Tiselius[1] appartus with the Longsworth[2, 3] optical arrangement.

ELECTROPHORESIS EXPERIMENTS

The serums were dialyzed against large volumes of phosphate buffer of pH 7.7 and of 0.2 ionic strength. The buffer was composed of 0.064 M Na_2HPO_4 and 0.008 M NaH_2PO_4 per liter. After dialysis all samples were adjusted to a protein concentration of 1 per cent. The electrophoresis runs were carried out at 0.5° and at a potential gradient of 5-6 volts per centimeter.

Data for the series are shown in table I and the patterns of four samples are illustrated in figures 1-4. Calculations were based on the patterns of the ascending boundaries because a very pronounced β-globulin disturb-

*Journal article No. 568, n.s., of the Michigan Agricultural Experiment Station, Section of Bacteriology.

[1]Tiselius, A.: A new apparatus for electrophoretic analysis of colloidal mixtures. Trans. Faraday Soc., xxxiii (1937), p. 524.

[2]Longsworth, L. G.: Modification of the "schlieren" method for use in electrophoretic analysis. J. Am. Chem. Soc., lxi (1939), p. 529.

[3]*Ibid.*: The observation of electrophoretic boundaries. Ann. New York Acad. Sci., xxxix (1939), p. 187.

TABLE I—Estimated Electrophoretic Mobilities* and Compositions* of Pullorum-Agglutinating Serums

SAMPLE	PLATE AGGLUTINATION	pH	MOBILITIES, u x 10^5					PROPORTION OF TOTAL PROTEIN (IN PER CENT)					COMPOSITION			
			Ax	A	α	β	γ	Ax	A	α	β	γ	A/G	α/A	β/A	γ/A
280a	Very delayed; doubtful	7.78		5.3	4.0	3.0	1.9	00	44	23	3	29	0.79	0.53	0.07	0.67
66a	Very delayed; doubtful	7.78		5.5	4.0	3.0	2.0	00	35	16	3	46	0.55	0.45	0.08	1.28
55a†	Slight, less than No. 66	7.78	7.0	5.6	4.0	3.1	?						?	0.47	0.11	0.00
69a	Slight, less than No. 66	7.78	6.6	5.2	3.8	2.8	1.9	5	37	6	10	42	.73	0.38	0.08	0.90
54a	Negative	7.78		5.3	3.9	3.0	2.0	00	33	15	5	46	0.49	0.47	0.16	1.41
173a‡	Negative	7.79		5.4	3.9	2.9	2.0	00	37	15	5	43	0.59	0.41	0.14	1.15
174a‡	Positive	7.79		5.2	3.7	2.8	1.7	00	27	13	2	58	0.37	0.47	0.08	2.16

*Calculated from the patterns of the ascending boundaries. The δ-boundary was not taken into account in these estimations.

†Both patterns possessed boundary disturbance.

‡Birds from another flock.

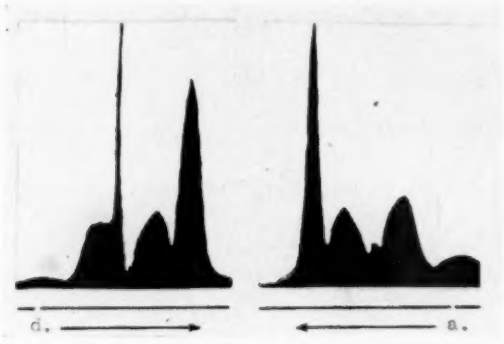

Fig. 1. Chicken serum 280a. Plate agglutination: delayed, doubtful.

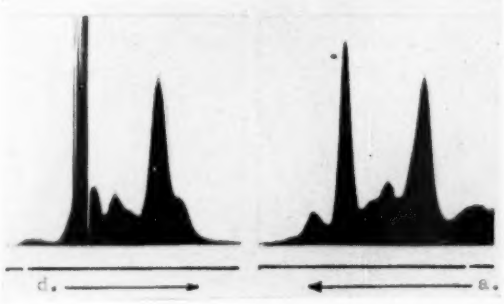

Fig. 2. Chicken serum 69a. Plate agglutination: slight, less than 280a.

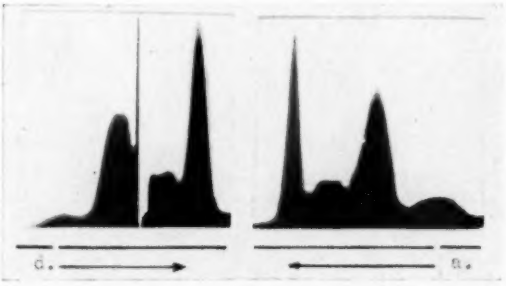

Fig. 3. Chicken serum 173a. Plate agglutination: negative.

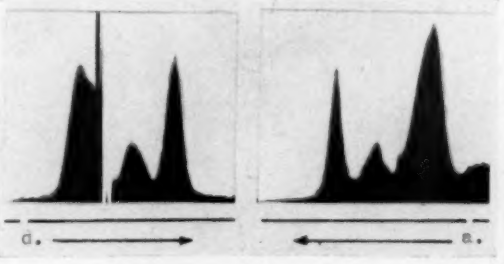

Fig. 4. Chicken serum 174a. Plate agglutination: strongly positive.

ance occurred in all the descending patterns. In the measurement of the areas of the patterns the δ-boundary was not considered.

DISCUSSION

Besides the β-globulin disturbance in the descending patterns and also on the ascending pattern of sample 55a, two samples, 69a (fig. 2) and 55a (not illustrated), differed strikingly from the others by the presence of a component, called A_x for convenience, traveling slightly faster than albumin. No reason is offered for this phenomenon other than the observation that negative or definitely positive serums showed no such component.

Table I shows that the serum samples possessed rather constant mobilities with respect to their various protein components. Such uniformity was not found for the relative proportion of total protein for each component. This fact may help to explain the inconsistency in the plate-agglutination test. Note that the average proportion of total protein (in per cent) for the negative samples was 35 for albumin, 15 for α-globulin, 5 for β-globulin, and 45 for γ-globulin. The albumin and γ-globulin components showed the greatest deviation in the positive and some of the doubtful samples. The column with the albumin-globulin ratios accentuates this difference. The γ/a ratio of the definitely positive serum was surprisingly high. The pattern of sample 174a (fig. 4) shows that γ-globulin alone accounted for 58 per cent of the total protein. This fact lends support to the supposition that pullorum antibody is associated with the γ-fraction. Although it is true that the γ-globulin concentration of the negative sample 173a (fig. 3) was much greater than that of the doubtful sample 280a (fig. 1), it must be remembered that the production of γ-globulin may be stimulated by antigens other than Salmonella. The doubtful serums all revealed irregularity in the relative concentrations of their protein fractions and in their A/G and γ/A ratios.

SUMMARY

The electrophoretic mobilities and compositions of several pullorum-agglutinating chicken serums were estimated. The relative mobilities of the protein fractions of

all samples were uniform, while the percentage concentrations of the protein fractions were relatively irregular for the doubtful and positive serums. The pullorum antibody is believed to be associated with the γ-fraction. The albumin concentration was decreased in serums of high titer.

ACKNOWLEDGMENT

The author thanks Dr. H. J. Stafseth of the Department of Bacteriology, Michigan State College, for suggesting and explaining the nature of the problem and for supplying the serum samples.

Ineffectiveness of Cow Urine in the Control of Eimeria Tenella Infection in Chickens*

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Vancouver, B. C.

COCCIDIOSIS, WHICH IS one of the most common and destructive diseases of poultry, is caused by a protozoan parasite which attacks various regions of the intestinal tract.[1-5] It may be acute, subacute or chronic in its manifestations. It is most injurious to growing chicks, and in an outbreak losses may vary from 15 per cent to 40 per cent. In older birds the infection usually assumes a chronic form, resulting in lowered egg production, gradual wasting away and high mortality. In response to requests from commercial poultrymen, an investigation was undertaken to determine the effects of cow urine and its constituents on coccidiosis in young chickens.

MATERIALS AND METHODS

A pure culture of *Eimeria tenella*, the organism causing cecal coccidiosis, was used in this study. Four birds were inoculated to increase the number and virulence of the coccidia. Three of them died and a stock culture was prepared from the cecal contents. In this preliminary experiment, as in all subsequent ones, a uniform procedure was followed. On the fifth and sixth days after inoculation, the droppings of the birds were examined for the appearance of blood. On the sixth and seventh days the dropping pans were found to be literally covered with pools of blood. The birds which died from the severe hemorrhage were subjected to postmortem examination and without exception showed typical lesions in the ceca. Those which did not die on the seventh or eighth day after inoculation were placed on a normal diet for an additional seven days, after which they were killed and examined. In a large number of cases they, too, showed lesions of coccidiosis, though less severe than in those which did not survive. In nearly all cases the diagnosis was confirmed by a microscopic examination of the cecal contents.

The birds used in this study were reared in battery brooders on wire floors and protected in so far as possible against accidental infection with coccidia. The average age of the birds used in the inoculation experiments was 6 to 9 weeks.

EXPERIMENTAL PROCEDURE

Experiment 1.—Four pens of eight birds each were placed on a growing ration and fed various amounts of urine in their drinking water. The birds were weighed weekly and the results are shown in table I. It is interesting to note that lasting harmful effects were not observed in chicks which had access to the drinking water containing as much as 75 per cent of urine for a four-week period.

Experiment 2.—An experiment was performed with four lots of eight birds each to determine the effect of urine on the virulence of coccidiosis. Twenty-five, 50 and 75 per cent of urine, respectively, was fed in the drinking water and one cage of birds was kept as a control. After one week on this diet each of the 32 birds was inoculated

*From the University of British Columbia.

[1]Becker, E. R.: Coccidia and coccidiosis. (Collegiate Press, Inc., Ames, Iowa, 1934.)

[2]Johnson, W. T.: J.A.V.M.A., lxx (1927), p. 560.

[3]Tyzzer, E. E.: J. Parasitol., xiii (1927), p. 215.

[4]*Ibid.*: Am. J. Hyg., x (1929), p. 269.

[5]*Ibid.*: Sci., lxxv (1932), p. 324.

TABLE I—Maintenance of Birds on Urine Diet

Days:	0	8			22			29			37			
% Urine	Av. Wt. (Gm.)	Av. Gain per Day (Gm.)	Total Av. Gain (Gm.)	Av. Wt. (Gm.)	Av. Gain per Day (Gm.)	Total Av. Gain (Gm.)	Av. Wt. (Gm.)	Av. Gain per Day (Gm.)	Total Av. Gain (Gm.)	Av. Wt. (Gm.)	Av. Gain per Day (Gm.)	Total Av. Gain (Gm.)	Av. Wt. (Gm.)	Total Av. Gain 37 Days
Control	272	14.9	119	391	19.0	266	657	20.4	143	800	15.4	123	923	651
25%	296	15.4	123	419	16.3	288	647	24.0	168	815	7.9	63	878	642
50%	289	12.1	97	386	11.0	154	540	20.2	141	681	13.8	111	792	503
75%	299	4.9	39	338	10.9	152	492	11.1	78	570	15.8	126	696	395

with 120,000 sporulated oöcysts of *E. tenella*. The results, shown in table II, indicated that urine lowered their resistance to coccidiosis.

Experiment 3.—Twelve birds were inoculated with varying amounts of *E. tenella* to determine the lethal dose. Postmortem examination showed considerable differentiation in the condition of the ceca. The lethal dose was found to be 120,000 sporulated oöcysts per chick.

Experiment 4.—Sixty-four birds were used in urine experiments. Thirty-two of them were placed under the same conditions as in experiment 2 to confirm the results as shown above. The remaining 32 birds were fed the same percentages of acid-hydrolyzed urine. The urine was subjected to acid hydrolysis as follows: The pH was adjusted to 1 with concentrated HCl, the liquid was hydrolyzed under 15 lb. of pressure for one hour in an autoclave, filtered and the pH adjusted to 7 with NaOH. The birds were fed 25, 50 and 75 per cent of this hydrolyzed urine in their drinking water. They were kept on this diet for a week, and at the end of this period all in the sections fed 50 and 75 per cent were dead. There are two possible explanations for this mortality: (1) either the hydrolysis converted some harmless constituent of the urine into a toxic substance or (2) the amount of NaCl introduced by the adjustment of the pH of the liquid was too high.

The surviving birds were inoculated, but owing to considerable difficulty experienced in maintaining the virulence of the *E. tenella* used in the experiment, the inoculations did not have the desired effect. In order to increase the virulence the culture was passed through several series of birds. The culture now appeared to be quite virulent, since a dose of 120,000 sporulated oöcysts proved fatal to the chicks.

Experiment 5.— Six urine fractions were prepared as follows: Six liters of urine was hydrolyzed under 15 lb. of pressure for one hour, after adjusting the pH to 1 with the aid of a Beckmann pH meter, as described in experiment 4. The hydrolyzed urine was then neutralized and subjected to vacuum distillation to concentrate it without danger of decomposition.

The hydrolyzed urine was evaporated to about 1 liter. It was then extracted four times with its own volume of ether, and the ether extract concentrated to a suitable amount. The urine so treated was reserved for experiment 5a and the concentrated ether fraction for experiment 5b.

In a similar manner 3 liters of unhydrolyzed fresh urine was concentrated *in vacuo* and treated with ether, the two fractions being reserved for experiments 5c and 5d.

After being allowed to stand for several days in an attempt to simulate practical conditions, a third lot of urine was similarly treated for experiments 5e and 5f. In addition, one pen was fed 75 per cent of urine in the drinking water, and one pen was reserved as a control.

Eight lots of eight birds each were then

TABLE II—Effect of Urine on Birds Inoculated with E. Tenella

% Urine	No. Birds	No. Dead	No. Survived	% Mort.
0 control	8	0	8	0
25	8	6	2	75
50	8	5	3	62
75	8	4	4	50

TABLE III—The Effect of Urine Fractions on Avian Coccidiosis

EXPERIMENT	CONDITIONS	NO. OF BIRDS	NO. INOCULATED	NO. DEAD	NO. SURVIVING	% MORTALITY
5a	Ether insoluble hydrolyzed	8	8	7	1	88
5b	Ether soluble hydrolyzed	8	8	6	2	75
5c	Ether insoluble unhydrolyzed	8	8	6	2	75
5d	Ether soluble unhydrolyzed	8	8	5	3	62
5e	Ether insoluble unhydrolyzed	8	8	6	2	75
5f	Ether soluble unhydrolyzed	8	8	5	3	62
5g	75% urine	8	8	8	0	100
5h	Control	8	8	4	4	50

placed on a half wheat, half mash diet into which the above six fractions had been incorporated. On the basis of a two-week feed, calculations were made of the amounts of urine fractions necessary to correspond to 75 per cent of urine fed in the drinking water. The birds were fed for seven days on this diet and then inoculated with 120,000 sporulated oöcysts each. Being uniformly negative, the results of this experiment, summarized in table III, confirmed in a general way those of the preceding ones. In each case the treated birds did not survive to the same extent as the controls.

Experiment 6.—This experiment was carried out to determine the effect of feeding various levels of urea on avian coccidiosis, as follows:

Experiment 6a: To 10 lb. of feed was added 91 Gm. of urea, constituting 2 per cent of the total.

Experiment 6b: To 10 lb. of feed was added 182 Gm. of urea—4 per cent of total.

Experiment 6c: To 15 lb. of feed was added 411 Gm. of urea—6 per cent of total.

The birds were fed on this diet for the usual seven-day period, carrying through a control lot at the same time, and then inoculated with 120,000 oöcysts of *E. tenella*. During the feeding period the droppings were much more liquid than usual, particularly in the higher percentages of urea, suggesting that the compound exerted a diuretic action. As shown in table IV, however, the results of the experiment did not indicate any advantage in feeding urea.

DISCUSSION

The experimental data recorded in this report were based upon inoculation experiments of chicks with *E. tenella*. This species of coccidium is responsible for the so-called cecal type of coccidiosis, which is one of the most virulent affecting poultry. However, six other species of coccidia are known to affect poultry. With one exception these species generally cause a chronic condition. The results of infection with the latter species sometimes do not become apparent for two or three months. The effect of cow urine on these types of coccidial infection has not been investigated in this research.

The opinion that urine has an inhibiting effect on coccidiosis, while not borne out in our research, may be derived from observations made by commercial poultrymen under farm conditions quite different from the

TABLE IV—The Effect of Urea on Avian Coccidiosis

EXPERIMENT	CONDITIONS	NO. OF BIRDS	NO. INOCULATED	NO. DEAD	NO. SURVIVING	% MORTALITY
6a	2% urea	8	8	5	3	62
6b	4% urea	8	8	7	1	88
6c	6% urea	8	8	5	3	62
6d	Control	8	8	5	3	62

experimental conditions under which direct inoculation is carried out.

SUMMARY

1) In order to determine the effect of cow urine and its constituents on the course and development of coccidiosis in chickens, several groups of chicks were inoculated with *Eimeria tenella* at the rate of 120,000 sporulated oöcysts per chick.

2) The inoculated chicks were studied to determine the effects of (a) urine on growth, (b) urine on coccidiosis, (c) varying amounts of culture, (d) hydrolyzed and unhydrolyzed urine, (e) urine fractions and (f) urea.

3) Favorable differences were not observed in the virulence of coccidiosis in the treated and control groups.

4) It was thus established that cow urine, various fractions of the urine, and urea did not protect growing chicks against lethal doses of *E. tenella*.